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# THE HARVEY LECTURES

# THE HARVEY LECTURES

DELIVERED UNDER THE AUSPICES OF
The HARVEY SOCIETY of NEW YORK
1945-1946

UNDER THE PATRONAGE OF THE NEW YORK
ACADEMY OF MEDICINE

BY

DR. JAMES A. SHANNON
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DR. GEORGE WALD
DR. MAX DELBRÜCK
DR. STAFFORD L. WARREN
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*SERIES XLI*

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# THE HARVEY SOCIETY

## A SOCIETY FOR THE DIFFUSION OF KNOWLEDGE OF THE MEDICAL SCIENCES

## CONSTITUTION

### I

This Society shall be named the Harvey Society.

### II

The object of this Society shall be the diffusion of scientific knowledge in selected chapters in anatomy, physiology, pathology, bacteriology, pharmacology, and physiological and pathological chemistry, through the medium of public lectures by men who are workers in the subjects presented.

### III

The members of the Society shall constitute three classes: Active, Associate, and Honorary members. Active members shall be laboratory workers in the medical or biological sciences, residing in the City of New York, who have personally contributed to the advancement of these sciences. Associate members shall be meritorious physicans who are in sympathy with the objects of the Society, residing in the City of New York. Members who leave New York to reside elsewhere may retain their membership. Honorary members shall be those who have delivered lectures before the Society and who are neither Active nor Associate members. Associate and Honorary members shall not be eligible to office, nor shall they be entitled to a vote.

Members shall be elected by ballot. They shall be nominated to the Executive Committee and the names of the nominees shall accompany the notice of the meeting at which the vote for their election will be taken.

## IV

The management of the Society shall be vested in an Executive Committee to consist of a President, a Vice-President, a Secretary, a Treasurer, and three other members, these officers to be elected by ballot at each annual meeting of the Society to serve one year.

## V

The Annual Meeting of the Society shall be held at a stated date in January of each year at a time and place to be determined by the Executive Committee. Special meetings may be held at such times and places as the Executive Committee may determine. At all meetings ten members shall constitute a quorum.

## VI

Changes in the Constitution may be made at any meeting of the Society by a majority vote of those present after previous notification to the members in writing.

# OFFICERS OF THE HARVEY SOCIETY

## OFFICERS

## 1946–1947

VINCENT DU VIGNEAUD, *President*
WADE W. OLIVER, *Vice-President*
COLIN M. MACLEOD, *Treasurer*
EDGAR G. MILLER, JR., *Secretary*

## COUNCIL

## 1946–1947

EARL T. ENGLE
L. EMMETT HOLT, JR.
HAROLD G. WOLFF

# FORMER OFFICERS OF THE HARVEY SOCIETY

## 1905–1906

| | |
|---|---|
| *President:* Graham Lusk | *Council:* |
| *Vice-President:* Simon Flexner | C. A. Herter |
| *Treasurer:* Frederic S. Lee | S. J. Meltzer |
| *Secretary:* George B. Wallace | Edward K. Dunham |

## 1906–1907

| | |
|---|---|
| *President:* Graham Lusk | *Council:* |
| *Vice-President:* Simon Flexner | C. A. Herter |
| *Treasurer:* Frederic S. Lee | S. J. Meltzer |
| *Secretary:* George B. Wallace | James Ewing |

## 1907–1908

| | |
|---|---|
| *President:* Graham Lusk | *Council:* |
| *Vice-President:* James Ewing | Simon Flexner |
| *Treasurer:* Edward K. Dunham | Theo. C. Janeway |
| *Secretary:* George B. Wallace | Philip H. Hiss, Jr. |

## 1908–1909

| | |
|---|---|
| *President:* James Ewing | *Council:* |
| *Vice-President:* Simon Flexner | Graham Lusk |
| *Treasurer:* Edward K. Dunham | S. J. Meltzer |
| *Secretary:* Francis C. Wood | Adolf Meyer |

## 1909–1910*

| | |
|---|---|
| *President:* James Ewing | *Council:* |
| *Vice-President:* Theo. C. Janeway | Graham Lusk |
| *Treasurer:* Edward K. Dunham | S. J. Meltzer |
| *Secretary:* Francis C. Wood | W. J. Gies |

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* At the Annual Meeting of May 18, 1909, these officers were elected. In publishing the 1909–1910 volume their names were omitted, possibly because in that volume the custom of publishing the names of the incumbents of the current year was changed to publishing the names of the officers selected for the ensuing year.

### 1910–1911

*President:* Simon Flexner
*Vice-President:* John Howland
*Treasurer:* Edward K. Dunham
*Secretary:* Haven Emerson

*Council:*
Graham Lusk
S. J. Meltzer
James Ewing

### 1911–1912

*President:* S. J. Meltzer
*Vice-President:* Frederic S. Lee
*Treasurer:* Edward K. Dunham
*Secretary:* Haven Emerson

*Council:*
Graham Lusk
James Ewing
Simon Flexner

### 1912–1913

*President:* Frederic S. Lee
*Vice-President:* Wm. H. Park
*Treasurer:* Edward K. Dunham
*Secretary:* Haven Emerson

*Council:*
Graham Lusk
S. J. Meltzer
Wm. G. MacCallum

### 1913–1914

*President:* Frederic S. Lee
*Vice-President:* Wm. G. MacCallum
*Treasurer:* Edward K. Dunham
*Secretary:* Augustus B. Wadsworth

*Council:*
Graham Lusk
Wm. H. Park
George B. Wallace

### 1914–1915

*President:* Wm. G. MacCallum
*Vice-President:* Rufus I. Cole
*Treasurer:* Edward K. Dunham
*Secretary:* John A. Mandel

*Council:*
Graham Lusk
Frederic S. Lee
W. T. Longcope

### 1915–1916

*President:* George B. Wallace*
*Treasurer:* Edward K. Dunham
*Secretary:* Robert A. Lambert

*Council:*
Graham Lusk
Rufus I. Cole
Nellis B. Foster

---

* Dr. William G. MacCallum resigned after election. On Doctor Lusk's motion Doctor George B. Wallace was made President—no Vice-President was appointed.

### 1916–1917

*President:* George B. Wallace
*Vice-President:* Rufus I. Cole
*Treasurer:* Edward K. Dunham
*Secretary:* Robert A. Lambert

*Council:*
Graham Lusk*
W. T. Longcope
S. R. Benedict
Hans Zinsser

### 1917–1918

*President:* Edward K. Dunham
*Vice-President:* Rufus I. Cole
*Treasurer:* F. H. Pike
*Secretary:* A. M. Pappenheimer

*Council:*
Graham Lusk
George B. Wallace
Frederic S. Lee
Peyton Rous

### 1918–1919

*President:* Graham Lusk
*Vice-President:* Rufus I. Cole
*Treasurer:* F. H. Pike
*Secretary:* K. M. Vogel

*Council:*
Graham Lusk
James W. Jobling
Frederic S. Lee
John Auer

### 1919–1920

*President:* Warfield T. Longcope
*Vice-President:* S. R. Benedict
*Treasurer:* F. H. Pike
*Secretary:* K. M. Vogel

*Council:*
Graham Lusk
Hans Zinsser
Frederic S. Lee
George B. Wallace

### 1920–1921†

*President:* Warfield T. Longcope
*Vice-President:* S. R. Benedict
*Treasurer:* A. M. Pappenheimer
*Secretary:* Homer F. Swift

*Council:*
Graham Lusk
Frederic S. Lee
Hans Zinsser
George B. Wallace

---

* Doctor Lusk was made Honorary permanent Counsellor.

† These officers were elected at the Annual Meeting of May 21, 1920, but were omitted in the publication of the 1919–1920 volume.

### 1921–1922

*President:* Rufus I. Cole
*Vice-President:* Stanley R. Benedict
*Treasurer:* A. M. Pappenheimer
*Secretary:* Homer F. Swift

*Council:*
Graham Lusk
Hans Zinsser
H. C. Jackson
W. T. Longcope

### 1922–1923

*President:* Rufus I. Cole
*Vice-President:* Hans Zinsser
*Treasurer:* Charles C. Lieb
*Secretary:* Homer F. Swift

*Council:*
Graham Lusk
W. T. Longcope
H. C. Jackson
S. R. Benedict

### 1923–1924

*President:* Eugene F. DuBois
*Vice-President:* Homer F. Swift
*Treasurer:* Charles C. Lieb
*Secretary:* George M. Mackenzie

*Council:*
Graham Lusk
Alphonse R. Dochez
David Marine
Peyton Rous

### 1924–1925

*President:* Eugene F. DuBois
*Vice-President:* Peyton Rous
*Treasurer:* Charles C. Lieb
*Secretary:* George M. Mackenzie

*Council:*
Graham Lusk
Rufus I. Cole
Haven Emerson
Wm. H. Park

### 1925–1926

*President:* Homer F. Swift
*Vice-President:* H. B. Williams
*Treasurer:* Haven Emerson
*Secretary:* George M. Mackenzie

*Council:*
Graham Lusk
Eugene F. DuBois
Walter W. Palmer
H. D. Senior

## 1926–1927

*President:* Walter W. Palmer
*Vice-President:* Wm. H. Park
*Treasurer:* Haven Emerson
*Secretary:* George M. Mackenzie

*Council:*
Graham Lusk
Homer F. Swift
A. R. Dochez
Robert Chambers

## 1927–1928

*President:* Donald D. Van Slyke
*Vice-President:* James W. Jobling
*Treasurer:* Haven Emerson
*Secretary:* Carl A. L. Binger

*Council:*
Graham Lusk
Russell L. Cecil
Ward J. MacNeal
David Marine

## 1928–1929

*President:* Peyton Rous
*Vice-President:* Horatio B. Williams
*Treasurer:* Haven Emerson
*Secretary:* Philip D. McMaster

*Council:*
Graham Lusk
Robert Chambers
Alfred F. Hess
H. D. Senior

## 1929–1930

*President:* G. Canby Robinson
*Vice-President:* Alfred F. Hess
*Treasurer:* Haven Emerson
*Secretary:* Dayton J. Edwards

*Council:*
Graham Lusk
Alfred E. Cohn
A. M. Pappenheimer
H. D. Senior

## 1930–1931

*President:* Alfred E. Cohn
*Vice-President:* J. G. Hopkins
*Treasurer:* Haven Emerson
*Secretary:* Dayton J. Edwards

*Council:*
Graham Lusk
O. T. Avery
A. M. Pappenheimer
S. R. Detwiler

### 1931–1932

*President:* J. W. Jobling
*Vice-President:* Homer W. Smith
*Treasurer:* Haven Emerson
*Secretary:* Dayton J. Edwards

*Council:*
Graham Lusk
S. R. Detwiler
Thomas M. Rivers
Randolph West

### 1932–1933

*President:* Alfred F. Hess
*Vice-President:* Haven Emerson
*Treasurer:* T. M. Rivers
*Secretary:* Edgar Stillman

*Council:*
Graham Lusk
Hans T. Clark
Walter W. Palmer
Homer W. Smith

### 1933–1934

*President:* Alfred F. Hess
*Vice-President:* Robert K. Cannan
*Treasurer:* Thomas M. Rivers
*Secretary:* Edgar Stillman

*Council:*
Stanley R. Benedict
Robert F. Loeb
Wade H. Brown

### 1934–1935

*President:* Robert K. Cannan
*Vice-President:* Eugene L. Opie
*Treasurer:* Thomas M. Rivers
*Secretary:* Randolph H. West

*Council:*
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B. S. Oppenheimer
Philip E. Smith

### 1935–1936

*President:* Robert K. Cannan
*Vice-President:* Eugene L. Opie
*Treasurer:* Thomas M. Rivers
*Secretary:* Randolph H. West

*Council:*
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Homer W. Smith
David Marine

### 1936–1937

*President:* Eugene L. Opie
*Vice-President:* Philip E. Smith
*Treasurer:* Thomas M. Rivers
*Secretary:* McKeen Cattell

*Council:*
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Martin H. Dawson
James B. Murphy

### 1937–1938

*President:* Eugene L. Opie
*Vice-President:* Philip E. Smith
*Treasurer:* Thomas M. Rivers
*Secretary:* McKeen Cattell

*Council:*
George B. Wallace
Martin H. Dawson
Herbert S. Gasser

### 1938–1939

*President:* Philip E. Smith
*Vice-President:* Herbert S. Gasser
*Treasurer:* Kenneth Goodner
*Secretary:* McKeen Cattell

*Council:*
Hans T. Clarke
James D. Hardy
William S. Tillett

### 1939–1940

*President:* Philip E. Smith
*Vice-President:* Herbert S. Gasser
*Treasurer:* Kenneth Goodner
*Secretary:* Thomas Francis, Jr.

*Council:*
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N. Chandler Foot
William S. Tillett

### 1940–1941

*President:* Herbert S. Gasser
*Vice-President:* Homer W. Smith
*Treasurer:* Kenneth Goodner
*Secretary:* Thomas Francis, Jr.

*Council:*
N. Chandler Foot
Vincent Du Vigneaud
Michael Heidelberger

### 1941–1942

*President:* Herbert S. Gasser
*Vice-President:* Homer W. Smith
*Treasurer:* Kenneth Goodner
*Secretary:* Joseph C. Hinsey

*Council:*
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Harold G. Wolff
Michael Heidelberger

### 1942–1943

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*Vice-President:* Thomas M. Rivers
*Treasurer:* Kenneth Goodner
*Secretary:* Joseph C. Hinsey

*Council:*
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## 1943–1944

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*Council:*
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## 1944–1945

*President:* ROBERT CHAMBERS
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*Treasurer:* COLIN MACLEOD
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*Council:*
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## 1945–1946

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# THE STUDY OF ANTIMALARIALS AND ANTIMALARIAL ACTIVITY IN THE HUMAN MALARIAS[1]

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NO PROBLEM in medicine for which a partial solution was already available has received such intensive study as have the malarias during the past four years. Nor has any problem, in such a short time, attracted such a diversity of investigative skills not previously applied to its solution. It is not surprising, then, that advances have been made towards an understanding of the biology of the malarial parasites and the natural histories of the diseases they cause, and towards the development of more effective means with which to combat their hazards.

The data to be discussed are derived from studies which have been conducted as a cooperative enterprise by the members of the Research Service and other groups as part of a program to improve antimalarial therapy sponsored by the Committee on Medical Research of the Office of Scientific Research and Development. Little of the information which has been acquired in this effort has as yet reached the scientific literature through ordinary channels. Consequently, it is well to emphasize, insofar as our own studies are concerned, that not only the experimental data collected, but also the reasoning which led to a progressive change in the orientation of the studies themselves, are in a true sense the contributions of the staff of the Research Service.[3] The work reported is that of one or another of the members of the Research Service, of the Research Service as a whole, or of other groups working under the general auspices of the Board for the Coordination of Malarial Studies.

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[1] Lecture delivered October 25, 1945. Based on work done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and New York University.

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Only a portion of the investigations on vivax malaria will be taken as the primary source of data for discussion, the ends of which are an understanding of the evolution of the problem and of the philosophy which underlies some of the experimental approaches which have been, or may be, used in searching for a solution.

The ultimate objective of the studies is a simple one, the improvement of antimalarial therapy. Complete success in this endeavor involves, first, an improvement in suppressive therapy; second, an improvement in the treatment of the clinical attack; third, the development of agents which will cure falciparum and vivax malaria at a well-tolerated dosage, and, finally, but perhaps of lesser practical importance, the development of agents which will prevent the inception of these diseases.

Each of these objectives presents a sizable problem in itself and one which would warrant intensive investigation over a considerable period of time. However, systematic studies were begun in the Research Service in 1942 on a rather modest scale and these were wholly exploratory in character. They were directed towards the development of reliable methods for the quantitative appraisal of antimalarial activity. This immediate objective required a consideration of the biology of the malarial infections and the factors which might be concerned with conditioning the therapeutic activity of antimalarial agents.

*The Biology of the Malarial Infection.* It would have been helpful had the exact disease mechanisms which underlie malarial infections been known. Information on the natural history of these diseases in the wholly susceptible individual was meagre in 1941 (1). However, it was adequate to formulate a reasonable working hypothesis (2, 3). This hypothesis had two advantages. It was amenable to experimental examination, and it could serve as a basis for the design of therapeutic tests which themselves would throw a light on the underlying disease mechanisms.

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[3] The group of investigators contributing to these studies has been large. In addition to the key personnel, Doctors D. P. Earle, Jr., B. B. Brodie, J. V. Taggart, R. W. Berliner, C. G. Zubrod, and W. J. Welch, the Service has been fortunate in having with it a number of additional physicians in the capacity of Residents in the Research Service for periods in excess of a year. Among the latter are: Doctors E. Bauman, F. S. Bigelow, W. J. Blake, T. C. Chalmers, T. J. Kennedy, P. Knowlton, W. E. Knox, M. Rosenfeld, and B. Wise.

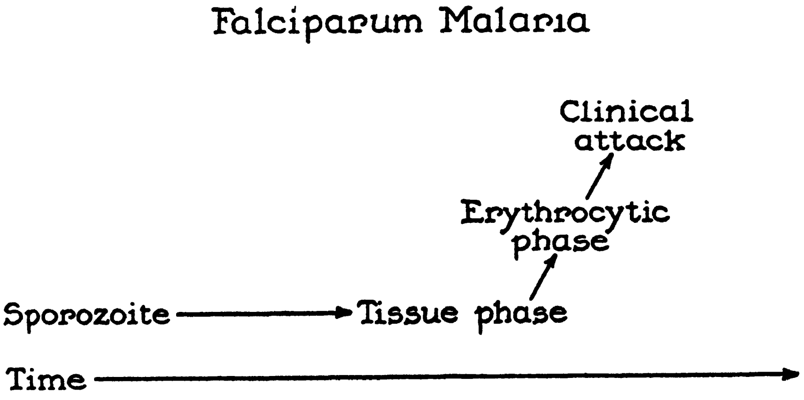

FIG. 1. An outline of a disease mechanism which seems adequate to explain the sequence of events in falciparum malaria.

*The Mosquito-induced Infection.* It seemed necessary to assume that the plasmodium, in each of the malaria infections, undergoes serial changes in the course of its residence in the human with the establishment of at least three discrete phases of development.

The malarias are acquired naturally when forms of the parasite known as sporozoites are introduced into the body incidental to the bites of infected anophelene mosquitoes (figure 1). This may be taken as the first stage of the disease and the first form of the parasite concerned with the human infection. It was also known that, while malaria may be easily induced by the transfer of blood containing parasites, the blood of a naturally infected individual is non-infectious for a period of some five to seven days following the deposition of sporozoites by the mosquito (4, 5, 6, 7, 8). During this period, i.e., the primary tissue phase of the disease, it was assumed that the sporozoites or the forms of parasites derived from them, reside in the body in sites other than the peripheral circulation, and undergo a developmental cycle which produces a third form of the parasite which is capable of invading the erythrocyte. The invasion of the erythrocytes initiates the third or erythrocytic phase of the disease. The latter form of the parasite grows, segments, and sporulates in the erythrocyte with the production of new parasites with similar potentialities. This part of the life history of the parasite is commonly called the asexual cycle. The process of multiplication of erythrocytic forms continues in the susceptible individual with a progressive increase in the number of parasites in the blood until a sufficient density is reached to precipitate the fever which is characteristic of the clinical attack.

The subsequent course of the disease in an individual is conditioned by the species of the plasmodium involved, by the presence or absence of natural and acquired immunity, and by whether an attempt is made to modify the course of the disease through the use of therapeutic agents (2). A discussion of the clinical characteristics of each of the malarias as affected by these variables is not germane to the present discussion. However, in the design of therapeutic tests, it was necessary to consider the mechanisms which might be responsible for the recurrence of clinical activity following the use of therapeutic agents. Little was known of these mechanisms other than that recurrences of clinical activity occur in a systematic fashion in vivax malaria and, to a lesser extent, in falciparum and quartan malaria (1).

Some believed these recurrences were due, in vivax malaria, to the simple persistence of resistant erythrocytic forms of the plasmodium. These investigators would postulate a disease mechanism for vivax malaria which now seems adequate for falciparum malaria, as summarized in figure 1, but with one addition. It was necessary to assume that the persistent erythrocytic forms were in locations within the body where, perhaps, they were not accessible to therapeutic agents. Others believed that, since it is reasonable to assume there is a primary tissue form of the parasite, it is equally reasonable to assume that a tissue form persists (4, 9). The latter appeared to be the more attractive hypothesis. In this view, providing therapy is adequate to interrupt the asexual cycle, the relapse is attributable to the potentialities of the persisting tissue forms to undergo periodic sporulation with the release of new lines of parasites capable of invading and growing in the erythrocytes and precipitating other bouts of clinical activity as discrete episodes in the course of the disease. Such evidence as has accrued since the beginning of these studies favors the acceptance of the mechanisms underlying falciparum and vivax malaria which are illustrated by figures 1 and 2 (10, 11, 12).

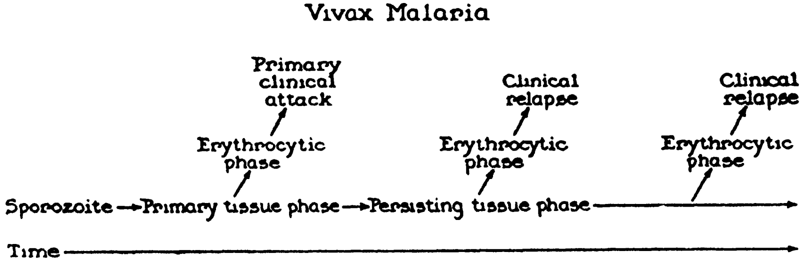

FIG. 2. An outline of a disease mechanism which seems adequate to explain the sequence of events in vivax malaria. Also, see figures 7 and 8.

It was apparent then, as now, that naturally acquired vivax malaria has a rather complex disease mechanism involving at least three stages of development of the parasite, exclusive of the sexual forms which are derived from the asexual cycle. Consequently, exclusive of the sexual forms, these are potentially at least three types of antimalarial activity: one involving the sporozoite; another, the primary tissue forms; and a third, the erythrocytic forms of the plasmodium. Also, since a persisting tissue phase of the disease was considered a strong possibility, there might be a fourth type of antimalarial activity which would become apparent in properly conducted studies.

*The Blood-induced Infection.* It seemed unwise to begin studies using an infection with a mechanism so complex that interpretable experimental results would not be assured. The more simple, blood-induced infection was used. This type of infection is established by the intravenous inoculation of blood containing parasites obtained from a patient with an active infection. These parasites are capable of growth, segmentation, sporulation, and invasion of new erythrocytes as in the naturally acquired infection. However, the disease is limited to the single erythrocytic phase. Consequently, when the asexual cycle is interrupted by adequate therapy, it is not characterized by the spontaneous recurrence of clinical activity. Such a simple disease mechanism has the advantage of offering the opportunity to study the characteristics of a single phase of the disease and a single type of antimalarial activity, i.e., suppressive type. Furthermore, since the mosquito-induced vivax infection can only manifest itself clinically by the establishment of an erythrocytic phase, it seemed reasonable to require complete information on this aspect of the infection and its response to drugs before proceeding to the study of the more complex disease.

*Natural History of the Standard Blood-induced Vivax Infection.* Malaria is induced in the standard blood-induced infection by the intravenous inoculation of blood containing 500,000 parasites into a wholly susceptible patient.[4] The infected blood is

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[4] The subjects used for the malarial infections were patients varying in age from 15 to 60 who were referred to the Research Service for fever therapy because of Central Nervous System Syphilis. Medical suitability for malarial therapy was based on a general medical examination which included the verification of the primary diagnosis and an evaluation of the neurological, cardiovascular, renal, and hepatic status of the patient, as well as a consideration of those intangibles which contribute to general physical fitness. Malaria was induced in those patients who were judged able to withstand a moderately severe infection without serious danger of a fatal termination. The amount of fever therapy administered in the ordinary case was approximately 75 hours of fever in excess of 103° F.

When the fever therapy was interrupted, as in a Class II or Class III derived from a patient with an active infection on the fourth or fifth day following the first day of fever. For present purposes, only two manifestations of the disease need be given consideration. These are: the fever and the parasitemia which are characteristic of the clinical attack.

The typical course of the blood-induced infection with the McCoy strain of *P. vivax*[5] is illustrated in terms of these two variables in figure 3. Following the inoculation of parasites, there is an incubation period of some days, in the present illustration, six, unaccompanied by clinical manifestations of the developing disease. Thereafter, a progressively increasing and fairly well-sustained fever is observed. This lasts for at least five days before the febrile pattern becomes intermittent and is characterized by well-defined malarial paroxysms. These occur daily during the early days of the infection but later become tertian in character. After a variable period of time, the febrile paroxysms diminish in severity, the temperature eventually stabilizing in the normal range. The clinical manifestations of the disease are then said to have terminated spontaneously. It is of particular importance to note that a spontaneous termina-

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therapeutic effect, it was continued to completion of the required amount by one or a combination of several procedures. These include the spontaneous or induced recurrence of clinical activity due to the same strain of plasmodium, reinfection with a different strain or species of plasmodium, or, the intravenous administration of triple typhoid vaccine in a manner which produces a sustained febrile paroxysm. These general procedures are important in that they permit the performance of therapeutic tests early in the course of the malaria and yet do not withhold adequate fever therapy from the patient.

[5] The McCoy strain of *P. vivax* was isolated in 1931 by Dr. Mark Boyd from a patient with a naturally occurring vivax infection presumably acquired in the vicinity of Tallahassee, Florida (13). It has since been carried by alternating anophelene human passages at the Tallahassee Malarial Station. The present offshoot of the strain was obtained by Dr. L. T. Coggeshall from Dr. Boyd for use at the Manhattan State Hospital in 1936. It was later transferred to Bellevue Hospital and finally to this Service in March 1941. Since 1936 the strain has been maintained by serial inoculation of infected blood from patient to patient.

tion of the disease due to the McCoy strain of *P. vivax* is not to be expected in any wholly susceptible individual until about 14 days after the first day of fever.[6]

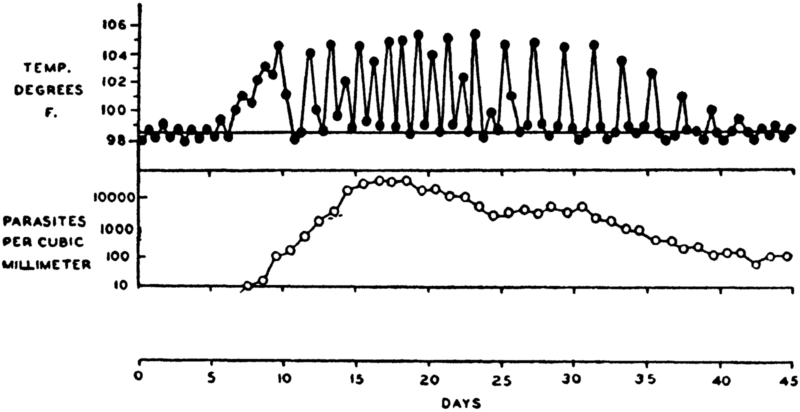

FIG. 3. A schematic presentation of the course of the fever and parasitemia in the standard blood-induced vivax infection (McCoy strain) when not influenced by the administration of an antimalarial.

Parasites are usually demonstrable by thick film a day or two before the first day of fever. Thereafter, they increase progressively in number until about the seventh to tenth day of fever.

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[6] An analysis of the data available indicates that with a standard inoculum of 500,000 parasites the mean duration of the pre-patent period is slightly in excess of 6 days. The duration of fever prior to spontaneous termination is usually in excess of 14 days (14). The latter finding is in rather sharp contrast to the previous results which have been reported for blood induced infections, using the McCoy strain of *P. vivax*. It has been stated that one may expect spontaneous terminations in as high as 35–40 per cent of individuals so infected, within a ten-day period (15). However, the latter results are perhaps attributable to the fact that the patients used in the previous studies were derived from an endemic malarious area and may not be considered to be wholly susceptible to the disease. It has been the experience of this service that previous exposure to any malarial infection usually shortens the duration of clinical activity. This point is deserving of special emphasis since the interpretation of a therapeutic effect requires a decision as to whether the termination of clinical activity is due to the administration of the therapeutic agent alone, or whether immune factors are in part responsible.

when they usually reach a maximum. The maximum is not well sustained and there is a progressive decline in density, anticipating somewhat, but roughly paralleling the decline in the intensity of the febrile paroxysms. However, with the complete loss of fever, i.e., with the spontaneous termination of the disease, a sizable density of the parasitemia is usually maintained for a considerable period of time. During this latter portion of the disease, the density of the parasitemia may fluctuate somewhat and mild febrile episodes are not uncommon.

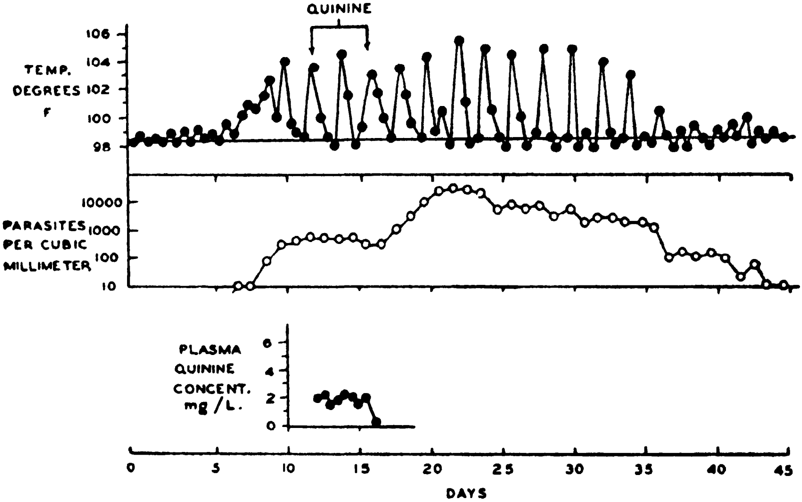

FIG. 4. A schematic presentation of the course of the fever and parasitemia in the standard blood-induced vivax infection (McCoy strain) in which the administration of quinine has produced a Class I therapeutic effect.

This description of the course of the disease is admittedly qualitative, but is quite adequate as a preliminary to the examination of the course of the disease when altered by the administration of a therapeutic agent which possesses a suppressive type of antimalarial activity. Providing the agent is administered in a stylized fashion, these alterations are amenable to classification into three categories through the application of rigid and objective criteria.

Figure 4 describes a Class I effect. As is routine with all therapeutic trials using McCoy vivax, administration of the antimalarial begins after 4 or 5 days of fever, and is continued in a manner calculated to maintain a reasonably stable plasma drug concentration for a 4-day period. The slight deceleration in the increase of parasitemia, in figure 4, which coincides with the sustained plasma quinine concentration of 2 mg./l. may be related to the antimalarial activity of quinine. However, such an effect is considered uncertain and placed in the category of Class I effects unless the parasitemia and fever are reduced by 50 per cent or more, and subsequently increase spontaneously.

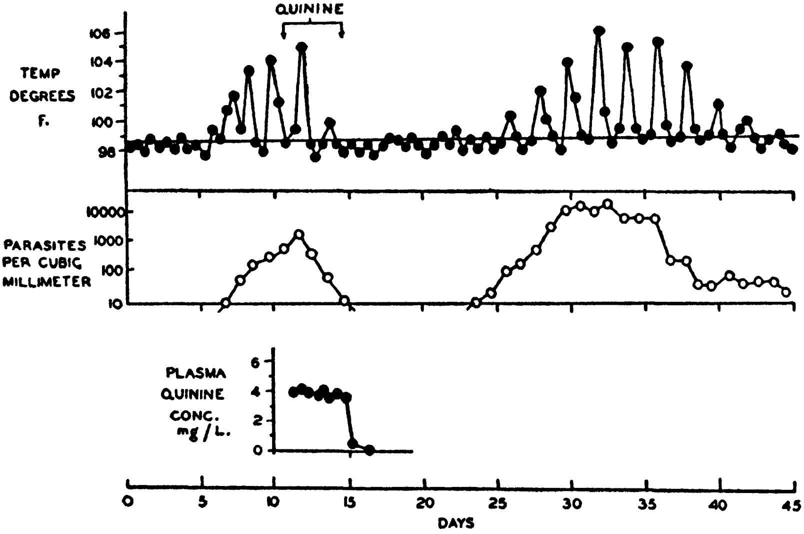

FIG. 5. A schematic presentation of the course of the fever and parasitemia in the standard blood-induced vivax infection (McCoy strain) in which the administration of quinine has produced a Class II theraputic effect.

The category of Class II effects includes all therapeutic effects wherein it is certain that the natural course of the disease has been altered but the effect has been insufficient to terminate the disease. This includes a partial effect on the parasitemia and fever, i.e., more than a 50 per cent reduction, and the more dramatic effect which is illustrated in figure 5. Here, the maintenance of a plasma quinine concentration, in the order of 3.0–4.0 mg. per liter, is accompanied by a rapid decline in the density of parasites and the intensity of fever, so that temperature is normal and parasites are no longer demonstrable by thick film on the day after the termination of therapy. However, the asexual cycle of the parasite has not been interrupted since there is a spontaneous recurrence of both parasitemia and fever nine days later.

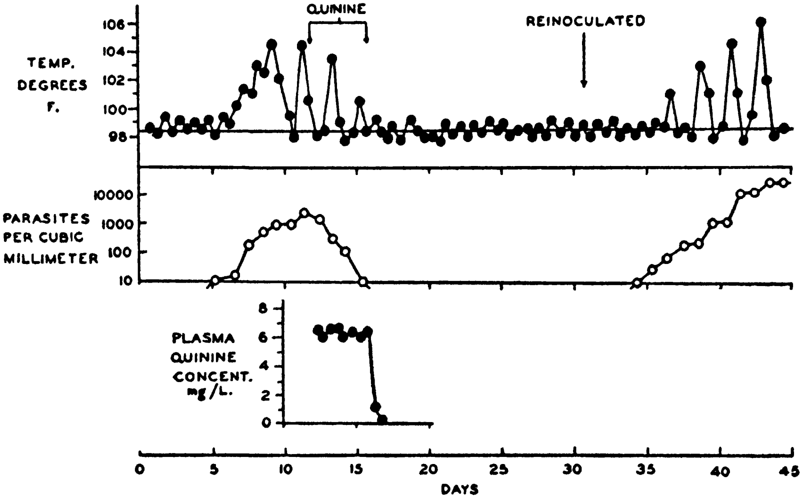

FIG. 6. A schematic presentation of the course of the fever and parasitemia in the standard blood-induced vivax infection (McCoy strain) in which the administration of quinine has produced a Class III therapeutic effect.

The category of Class III effects includes only those therapeutic effects which may be interpreted to indicate that the antimalarial administered has caused a complete interruption of the asexual cycle and, consequently, a termination of the infection. An illustration of such an effect is given in figure 6. Coincidental with a sustained plasma quinine concentration of some 6 mg. per liter, there is, as in the previous illustration, a rather sharp decline in parasitemia and fever. However, the parasites are absent for a period in excess of 14 days following the termination of therapy. Such a therapeutic result can be obtained solely as the result of the antimalarial activity of quinine or can be due in part to natural or acquired immunity. Consequently, the role of immunity must be excluded as an important factor conditioning the result in each therapeutic test. This is accomplished by two devices. First, the therapeutic agent is administered early in the course of the disease, beginning on the 4th or 5th day of the febrile episode and before a significant amount of immunity has been developed.[6] Second, each patient who has shown a therapeutic result which is potentially a Class III effect, is examined for continued susceptibility by studying the response to the reinoculation of parasites. Should parasitemia and fever develop within the normal time following the reinoculation of parasites, then it is concluded the patient has a continuing susceptibility. Such a positive result, as in this illustration, is taken as an indication that the termination of the initial clinical attack was due to the drug administered, and that the therapeutic test is not seriously complicated by immunity.

Some additional comment is warranted concerning the separation of Class II and Class III effects. This relates to the time selected for the reinoculation procedure. It has been demonstrated, in all but the exceptional patient, that an observation period of 14 days following the last significant plasma drug concentration is adequate in McCoy vivax for the spontaneous recurrence of parasites if such a recurrence is to be expected. However, with drugs such as quinacrine and the 4-aminoquinolines which persist in the body for considerable periods of time, the interval between the termination of therapy and reinoculation must be longer. The beginning of the 14-day observation period must be delayed in each case until the plasma drug concentration has reached a level which is either insignificant or is known to have no therapeutic effect. Note should also be taken of the fact that the duration of the observation period which is necessary to separate Class II from Class III effects varies from strain to strain in any species of plasmodium as well as from species to species. It must be determined experimentally. For example, it has been found in the Chesson strain of *P. vivax* (16) that a 21-day period of observation is necessary in blood-induced infections if essentially all of the spontaneous recurrences are to be included.

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[6] See footnote 6 on page 50.

These techniques were used to standardize the susceptibility of the blood-induced McCoy vivax malaria to antimalarial agents. Quinine was selected as the primary standard of reference because it seemed logical to use the one drug about which a great deal was known, because the development of chemical methods for its estimation seemed a simple matter (17), and because early observations indicated that a therapeutically active concentration is maintained in the plasma not more than 12 hours after therapy is terminated (14).

Data bearing on this standardization, collected from a series of

TABLE 1

*The Relationship Between Dosage, the Mean Plasma Level of Quinine (4 Days) and Its Therapeutic Effect in the Standard Blood-induced Therapeutic Test with McCoy Vivax Malaria*

These observations were made in the spring and summer of 1942.

| Patient | Drug dosage | | Mean plasma conc. | Result | | |
|---|---|---|---|---|---|---|
| | Initial | Daily | | Class I | Class II | Class III |
| | *g.* | *g.* | *mg./l.* | | | |
| DI | 0.3 | 0.36 | 6.2 | | | + |
| PE | 0.15 | 0.36 | 5.8 | | | + |
| ME | 0.3 | 0.36 | 5.1 | | | + |
| FA | 0.3 | 0.24 | 4.3 | | | + |
| BA | 0.3 | 0.36 | 3.9 | | + | |
| LY | 0.18 | 0.21 | 3.7 | | + | |
| DE | 0.06 | 0.3 | 3.0 | | + | |
| GO | 0.09 | 0.12 | 2.5 | | + | |
| MA | 0.09 | 0.12 | 2.3 | | + | |
| SI | 0.18 | 0.12 | 2.2 | + | | |
| SH | 0.18 | 0.12 | 1.8 | + | | |
| CA | 0.09 | 0.12 | 1.4 | + | | |

patients in the spring and summer of 1942, are summarized in table 1. It is apparent that the correlation between the mean plasma quinine concentration and the therapeutic effect is excellent; concentrations below 2 mg. per liter yielding Class I effects; between 2 and 4 mg. per liter, Class II effects; and 5 mg. per liter or higher, Class III effects. The correlation between the oral dosage of quinine and the therapeutic effect is poor. It was tentatively concluded from these data that the antimalarial activity of quinine is a simple expression of its concurrent plasma concentration, and that this activity is amenable to simple evaluation. The simplicity of such an evaluation is to be emphasized since the ability to relate an antimalarial effect to a given concentration of active principle in the plasma permits a definitive assay of activity with a very small number of therapeutic trials.[7] This is an essential for the serial examination of any large number of potential antimalarials.

Before an experimental preparation, such as that described above, can be used in the study of the comparative antimalarial activity of other substances and the results accepted with finality, it is necessary to know the extent to which the therapeutic test object has a stable susceptibility to the antimalarial activity of quinine. Information bearing on this point was obtained in a second standardization performed during the summer and fall of 1943, with a result essentially the same as that observed the previous year (table 2). It may be concluded that the susceptibility of this infection to the antimalarial activity of quinine is a stable

[7] It would have been possible to standardize the susceptibility of this infection in terms of the oral dosage of quinine required to produce a given therapeutic effect. However, such a standardization would have required a considerable increase in the number of therapeutic test runs before a definitive standardization was achieved. This is because of the poor correlation between oral dosage of quinine and plasma quinine concentration achieved and maintained (see figure 11). Such a procedure would have been even more difficult if not impossible with certain other drugs which have been examined since it is always necessary to know the duration of time an active concentration persists in the body subsequent to the termination of therapy before the drug-free observation period can be estimated. This is discussed in relation to the separation of Class II and Class III effects (see pp. 52–53).

TABLE 2

*The Relationship Between Dosage, the Mean Plasma Level of Quinine (4 Days) and Its Therapeutic Effect in the Standard Blood-induced Therapeutic Test with McCoy Vivax Malaria*

These observations were made in the summer and fall of 1943.

| Patient | Drug dosage | | Mean plasma conc. | Result | | |
|---|---|---|---|---|---|---|
| | Initial | Daily | | Class I | Class II | Class III |
| | *g.* | *g.* | *mg./l.* | | | |
| IR | 1.0 | 0.36 | 8.9 | | | + |
| GU | 0.3 | 0.36 | 8.5 | | | + |
| KL | 0.5 | 0.60 | 6.5 | | | + |
| GR | 0.5 | 0.60 | 6.1 | | | + |
| SE | 0.3 | 0.36 | 6.1 | | | + |
| BE | 0.3 | 0.48 | 5.0 | | | + |
| PI | 0.3 | 0.36 | 5.0 | | + | |
| KE | 0.3 | 0.48 | 4.1 | | | + |
| MA | 0.3 | 0.36 | 3.5 | | + | |
| TA | 0.3 | 0.36 | 3.4 | | + | |
| HO | 0.3 | 0.45 | 3.0 | | + | |
| TE | 0.12 | 0.12 | 2.9 | | + | |
| MO | 0.12 | 0.12 | 2.9 | | + | |
| DO | 0.3 | 0.36 | 2.9 | | + | |
| AN | 0.3 | 0.54 | 2.9 | | + | |
| CI | 0.3 | 0.36 | 2.8 | | + | |
| HU | 0.12 | 0.12 | 1.9 | + | | |
| JA | 0.12 | 0.12 | 1.2 | + | | |

characteristic of the strain, when the estimation is carried out in a highly stylized manner. As a corollary to this, it was also concluded that the assay of comparative antimalarial activity of any series of compounds is not only possible, but should be a rather simple matter.

*The Mosquito-induced Infection.* It seemed necessary to inquire, next, into the meaning of these experimental results in terms of naturally acquired vivax malaria. The many studies which had been reported in the literature suggested that the daily administration of 1.0 gram of one of the soluble salts of quinine for a period of 7 days is usually sufficient to terminate a clinical attack of vivax malaria (18). The daily administration of 1.0 gram of quinine sulfate in the ordinary sugar-coated pill produces equilibrium plasma drug concentrations which range from below 5 to 9 mg. per liter (14). Consequently, it was possible to conclude that the McCoy strain, when properly used in a blood-induced infection, is not peculiarly susceptible to the suppressive type of antimalarial activity of this drug since a minimal plasma drug concentration of 5 mg. per liter must be maintained for 4 days to interrupt the asexual cycle. This conclusion was important since it followed that an antimalarial judged to be promising on the basis of the blood-induced test would not be found to be without promise when tested under the more diverse conditions obtaining in the field.

However, for other reasons, it was decided to extend the experiments with quinine to include an examination of its antimalarial activity in the mosquito-induced infections due to the same strain of *P. vivax.* Therapeutic trials were run in essentially the same fashion as were those with a blood-induced infection, except for the manner of inducing the infection and the duration of the follow-up period. The data in table 3 summarize the experimental results which were obtained in the initial clinical attacks of a series of these infections.

These results can only mean that the erythrocytic phase of the disease is equally susceptible to quinine, whether derived from a mosquito-induced infection, or from the transfer of infected blood. Or, stated in more general terms, the erythrocytic forms of the parasite would appear to have the same chemotherapeutic characteristics in the two situations. Consequently, the relapse, which is a fairly consistent feature of the mosquito-induced infection, but which does not occur in the blood-induced infection, cannot be reasonably attributed to the persistence of some portion of the erythrocytic phase of the disease. Rather, the relapse must be due to some other form of the parasite not present in the blood-induced infection. This is assumed to be a form of the parasite which persists in the tissue.

TABLE 3

*The Relationship Between Dosage, the Mean Plasma Level of Quinine (4 Days) and Its Therapeutic Effect in Primary Attacks of Mosquito-induced McCoy Vivax Malaria*

**The therapeutic tests were performed in the same manner as those summarized in tables 1 and 2 with the exception that they were induced by bites from infected mosquitoes.**

| Patient | Drug dosage | | Mean plasma conc. | Result | | |
|---|---|---|---|---|---|---|
| | Initial | Daily | | Class I | Class II | Class III |
| | *g.* | *g.* | *mg./l.* | | | |
| FA | 1.0 | 1.8 | 13.1 | | | + |
| BL | 1.0 | 1.8 | 10.7 | | | + |
| DA | 1.0 | 1.8 | 9.8 | | | + |
| KA | 1.0 | 1.8 | 9.3 | | | + |
| OB | 1.0 | 1.8 | 8.6 | | | + |
| SC | 0.25 | 0.45 | 7.5 | | | + |
| LO | 0.18 | 0.36 | 6.7 | | | + |
| DO | 0.25 | 0.45 | 6.4 | | | + |
| KO | 0.18 | 0.36 | 5.2 | | | + |
| SI | 0.25 | 0.45 | 5.1 | | | + |
| SM | 0.25 | 0.45 | 4.3 | | + | |
| HO | 0.18 | 0.36 | 3.2 | | + | |
| PO | 0.18 | 0.36 | 3.1 | | + | |
| KI | 0.18 | 0.36 | 2.9 | | + | |
| BU | 0.18 | 0.36 | 2.0 | + | | |

Information concerning the potentialities of these tissue forms to release new lines of parasites which are capable of invading and growing in the erythrocytes can be gained from a study of the subsequent course of the mosquito-induced infection. The course of the disease (McCoy vivax) was followed closely for a period of 12 or more months in 18 patients (table 4). Each clinical attack was treated with more than sufficient quinine to interrupt the asexual cycle. It is apparent from these data that the initial relapse rate is reasonably high, being in excess of 60 per cent within the first year and that, characteristically, the first relapse occurs during the 8th or 9th month following the initial infection. These data indicate a rather definite pattern for the infection (cf. 4).

The sequence of events in a typical infection due to the McCoy strain of *P. vivax* is illustrated in figure 7. This shows the initial clinical attack, interrupted by quinine, with the persistence of the underlying tissue phase of the disease, quiescent as far as overt clinical manifestations are concerned for a period of 6 months when a second erythrocytic phase of the disease is established. Clinical activity which is usually seen at this time is undoubtedly due to the maturation of persisting tissue forms of the parasite which require this time before they are capable of releasing new transitional forms of parasites which invade the blood stream. Should the disease process be altered again by the administration of quinine or another therapeutic agent, then further relapses may be expected in varying numbers and at varying periods of time.

TABLE 4

*The Occurrence of First Relapses in a Series of Eighteen Patients with Mosquito-induced McCoy Vivax Malaria Who Were Observed Closely for a Period of 12 Months or More*

| | Duration of follow-up months | | | | |
|---|---|---|---|---|---|
| | 1–4 | 5–6 | 7–8 | 9–10 | 11 or more |
| No. of patients observed . | 20 | 20 | 19 | 18 | 18 |
| Cumulative relapse .. | 1 | 2 | 9 | 12 | 12 |

Very extensive data of this type have been collected by Coatney and his collaborators of the U.S.P.H.S. on another domestic strain of *P. vivax,* the St. Elizabeth's strain, which support this view of the underlying mechanism of the disease and add an additional and important fact (19). These investigators have demonstrated by subinoculation procedures that during the period of quiescence or latency, as is to be expected, the blood is completely free of an infectious agent. The belief that the persisting form of the parasite which is responsible for the relapse is in the tissues is considerably strengthened by these data, as is the initial premise concerning the overall disease mechanism which underlies the vivax infection.

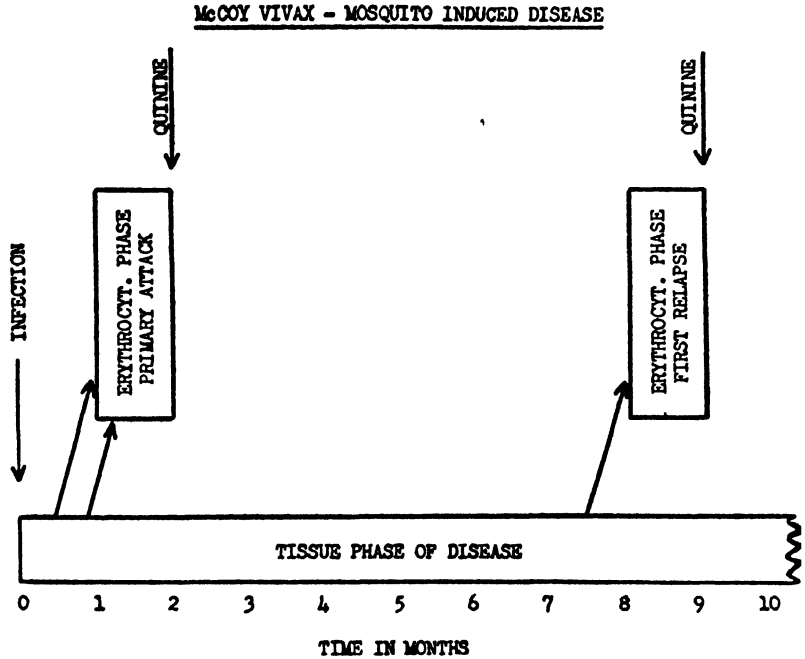

FIG. 7. A diagram of the underlying disease mechanism of mosquito-induced McCoy vivax malaria. Note particularly that the tissue phase of the disease persists during a period of some six months during which time there is no clinical evidence of the disease; also that the relapse occurs as a discrete episode in the course of the disease.

Examination of the detailed data available on our experimental subjects, those studied by Coatney, and those reported in the literature, indicates that quinine has an antimalarial activity in the established infection which, at well-tolerated doses, is suppressive in type and wholly limited to the erythrocytic forms of the parasite. Consequently, quinine or a drug with similar characteristics, can be used to define the activity of the underlying tissue phase of the disease with some precision.

This technique has been applied in a preliminary fashion to an exotic strain of *P. vivax* isolated by Young about a year ago (16). The Chesson strain of *P. vivax* produces a clinical disease which is similar to the usual type of vivax infection acquired in the Southwest Pacific. The early portion of the disease is similar in all characteristics to McCoy vivax. However, there is no long latent period between the primary attack and the relapse, as in the McCoy or St. Elizabeth vivax but, rather, one observes repeated clinical activity occurring in many individuals at relatively short intervals. These relapses may occur as early as seven days after the termination of quinine therapy, or a comparable period of time following the fall in plasma quinacrine concentration to an ineffective level. It was believed that this type of vivax malaria would be invaluable in the examination of drugs for curative action in vivax malaria since the occurrence of relapses in a short time would permit a tentative answer to be reached without requiring the long term follow-up which is necessary when a domestic strain of *P. vivax* is used.

Before the Chesson strain could be used with confidence in studies of this type, it was necessary to determine which of two possible mechanisms is responsible for the short-term renewals of clinical activity. Such recurrence could be due to the normal relapse mechanism as described above for the McCoy vivax. In this view the pattern of activity of the underlying tissue phase is such that it undergoes repeated short-term sporulations, each time releasing transitional forms of the parasite which are capable of invading the erythrocytes and precipitating a true relapse. It was also believed possible that the erythrocytic phase of the disease might be peculiarly resistant to the activity of antimalarials and the short-term renewals of clinical activity are the result of an inability of the drugs used to interrupt the asexual cycle. This question was studied by examining the susceptibility of the erythrocytic phase of the disease to quinine and quinacrine in blood-induced Chesson malaria. Data bearing on this question are summarized in table 5. The critical plasma quinine level required to produce a Class III effect is somewhat higher than for McCoy vivax and must be maintained for six, rather than four days. These data indicate that the erythrocytic phase of Chesson vivax is somewhat less susceptible to quinine than was previously found for McCoy vivax malaria. However, this greater resistance is not sufficient to prevent the interruption of the asexual cycle by quinine or quinacrine when administered in the usual manner. It may be concluded from these data that the short-term recurrence of activity which is the rule in mosquito in-

TABLE 5

*The Relationship Between Dosage, the Mean Plasma Concentration of Quinine (Six Days) and Its Therapeutic Effect in Trials Utilizing Blood-induced Chesson Vivax Malaria*

These trials differ from those described for the McCoy strain of *P. vivax* in two respects. Quinine was administered in each test for six rather than for four days since with administration limited to four days, daily doses of 1.8 grams quinine and concentration of plasma quinine as high as 12 mg. per liter do not produce Class III effects in a systematic manner. The period of observation following therapy and before reinoculation was extended to 21 days.

| Patient | Drug dosage | | Mean plasma conc. | Result | | |
|---|---|---|---|---|---|---|
| | Initial | Daily | | Class I | Class II | Class III |
| | *g.* | *g.* | *mg./l.* | | | |
| DE | 0.6 | 1.8 | 14.6 | | | + |
| SE | 1.0 | 1.8 | 12.8 | | | + |
| GE | 1.5 | 2.4 | 12.6 | | | + |
| MC | 1.5 | 1.8 | 11.8 | | | + |
| WE | 0.3 | 0.9 | 11.5 | | | + |
| HU | 0.6 | 1.8 | 8.6 | | | + |
| ME | 1.5 | 2.4 | 8.0 | | | + |
| AN | 0.18 | 0.36 | 6.6 | | | + |
| OA | 1.0 | 2.4 | 6.0 | | + | |
| PE | 1.0 | 2.4 | 3.6 | | + | |

duced Chesson malaria is in fact due to a mechanism which is characteristic of the relapse in vivax malaria, as illustrated in figure 8.

*The examination for antimalarial activity.* This background of information on the natural history of vivax malaria permitted

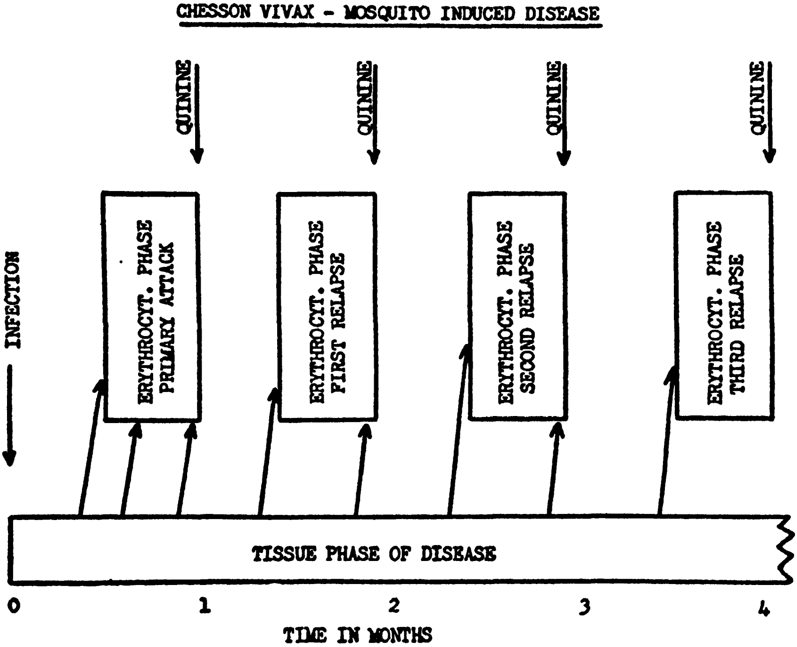
CHESSON VIVAX - MOSQUITO INDUCED DISEASE
QUININE
QUININE
QUININE
QUININE
INFECTION
ERYTHROCYT. PHASE PRIMARY ATTACK
ERYTHROCYT. PHASE FIRST RELAPSE
ERYTHROCYT. PHASE SECOND RELAPSE
ERYTHROCYT. PHASE THIRD RELAPSE
TISSUE PHASE OF DISEASE
0
1
2
3
4
TIME IN MONTHS

FIG. 8. A diagram of the underlying disease mechanism of mosquito-induced Chesson vivax malaria. Note particularly that, contrary to McCoy vivax malaria, relapses occur at relatively short intervals even though, as in McCoy vivax, each may be considered to be a discrete episode occurring in the course of the disease. The pattern of the relapses with respect to time in such a situation is determined in part by the character of the therapy.

the design of therapeutic tests which make possible the examination of antimalarial agents for each of three types of activity, i.e., upon the primary tissue phase of the disease, upon the erythrocytic phase of the disease, and upon the persisting tissue phase of the disease. As a convenience, these types of activity have been designated as prophylactic, suppressive, and curative, respectively.

Ideally, the study of the prophylactic action of a drug should be conducted in a manner whereby its therapeutic activity is limited to the first six or seven days of the infection. The persistence of a therapeutically active concentration beyond this time may be expected to alter the normal course of the infection through a suppressive effect upon the erythrocytic forms of the parasite. This may be reflected in the absence of the initial clinical attack with our domestic strains of *P. vivax* or in the delay in the initial clinical attack with a strain having the characteristics of Chesson vivax (11, 12, 19). When a prophylactic test is interfered with by the persistence of a suppressive drug, other techniques may be used to determine whether the suppressive has, in addition, a prophylactic action. Subinoculation procedures are invaluable for this purpose. For example, Fairley (12) has demonstrated rather clearly that the currently recommended regimens of suppressive quinacrine therapy in no way delay the time of appearance of the erythrocytic forms of the parasite. Consequently, it may be concluded that quinacrine, in these doses, does not alter the initial phase of the disease, and has no prophylactic action. Also, techniques have been developed which permit the concentration of parasites contained in a 10-ml. sample of blood into as little as 10 cubic mm. of volume (20). It has been shown, using a modification of this technique, that quinine and SN–7618 (21), in full therapeutic doses, have no effect on the initial phases of the disease (22).

Antimalarial activity of a suppressive nature being exerted against the erythrocytic forms of the parasite can be most simply evaluated through the use of a standard blood-induced infection, as previously described. Antimalarial activity of a curative nature can only be demonstrated providing therapeutic tests, which are performed in mosquito-induced infections, are conducted in a fashion which is known, on the basis of information derived from the blood-induced infection, to interrupt the asexual cycle of the parasite, and providing follow-up periods are sufficiently long to include a final estimation of relapse rates with the test drug as compared with the standard of reference.

*General Principles of Chemotherapy of the Malarias.* The discussion, so far, has been devoted to a consideration of the natural history of the vivax malarias, and the disease mechanisms which underlie their clinical manifestations insofar as these condition the examination of substances for antimalarial activity. It is equally important to have an appreciation of certain fundamental concepts which are applicable to any situation wherein a chemical agent is used to produce a given therapeutic effect (23).

The activity of any chemotherapeutic agent must result from its ability to participate in or interfere with some phase of biological activity. However, the specific, as well as the overall activity of the agent will be conditioned by those factors which determine its ability to reach the specific site of its action, the concentration it achieves at that site, and the length of time a biologically active concentration is maintained. It follows from this that the examination of the chemotherapeutic action of a compound will be facilitated by the use of experimental techniques which separate the factors which are directly related to the physiological disposition of the compound by the host, from those which are directly related to the action of the compound in any given biological system within the host.

For practical purposes, it has been assumed that this effect can be achieved in the malarias providing it is possible, first, to define the relationship between the concentration of an active agent in the plasma and its concurrent antimalarial activity and, second, to describe the operation of those processes which together determine the relationship between drug administration and plasma concentrations of the active material which are attained. Emphasis has been given to this view in the studies of antimalarials. This is partly because plasma is the medium of exchange of all substances as they are absorbed, distributed and degraded in the body and which, together with other factors, determine their rates of renal excretion. Consequently, in any given situation, the concentration of an antimalarial in the plasma may be taken as an integrated expression of the operation of these several processes. Furthermore, at least in the case of the erythrocytic phase of the disease, the concentration of an active substance in the plasma is that to which the parasitized erythrocytes are continuously exposed.[8] The intelligent study of the potentialities of any antimalarial agent, therefore, requires the availability of chemical methods to assay its concentration in biological tissues and fluids and at times, to follow its course of metabolism.

*Course of studies.* The studies on the therapeutic activity of antimalarial drugs during the past four years have developed in three distinct phases (3). The first phase of work, extending from the spring of 1942 to the winter and spring of 1943, was concerned with an examination and the development of a better usage of antimalarial agents already available. This was an effort which was the primary concern of our own Service and involved little in the way of help from cooperating laboratories in the national program. The second and third phases of the investigation were cooperative efforts on a national scale and dealt with the development and examination of new antimalarial agents.

The earlier work was conditioned largely by the early loss to the United Nations of their normal sources of supply of quinine, by the lack of an adequate stockpile of quinine, and by preliminary reports from the field which carried the suggestion that quinacrine was of little use in the routine suppression and treatment of malaria. Quinacrine was said to be unable to produce a prompt termination of the clinical attack, much less a cure in either falciparum or vivax malaria. In addition, it was reported

[8] Admittedly this is an overamplification since most highly active antimalarials which have been studied are extensively bound by the plasma proteins and many have apparent acid dissociation constants which are in the range where minor changes in the pH of plasma cause large shifts in the relationship between bound and free drug at any given total plasma concentration. Equilibria across membrane or at surfaces must be conditioned by the concentrations of the molecular species of unbound drug rather than by total plasma concentration of drug.

to be highly toxic and relatively ineffective when used for suppressive purposes. The immediate solution of the problem posed by the essential lack of any well-tolerated antimalarial agent of known activity was the result of two lines of investigation.

Cinchona alkaloids: It was first demonstrated that the early observers who studied the antimalarial activity of the various cinchona alkaloids (24) were essentially correct in their conclusions in that each of the four cinchona alkaloids has essentially the same overall antimalarial effect (14). This demonstration involved a series of studies on the antimalarial activity of the various cinchona alkaloids in the standard blood-induced infection. It was demonstrated with considerable probability that such activity, as with quinine, is a reflection of the concurrent plasma drug concentration of the alkaloid, and that the critical plasma concentration of each alkaloid which is required to produce a class III effect in the standard blood-induced infection varies greatly when one is compared to another (table 6). However, the oral dosage of each alkaloid required to produce such a critical concentration is of the same order of magnitude, being least for quinidine, and most, for cinchonine. This information was important because it placed the use of the cinchona alkaloids other than quinine on a sound basis and made large supplies of cinchona bark from Central and South American countries available for general use. These barks were very low

TABLE 6

*The Plasma Concentration of the Several Cinchona Alkaloids Which, When Maintained for Four Days, Are Accompanied by Class III Therapeutic Effects in the Standard Blood-induced Therapeutic Test Using the McCoy Strain of P. vivax.*

See footnote 12 for further information on cinchonine.

| | |
|---|---|
| Quinine | 5.0 mg. per liter |
| Quinidine | 1.0 mg. per liter |
| Cinchonidine | 3.0 mg. per liter |
| Cinchonine | 0.1 mg. per liter |

## TABLE 7

*The Relationship Between Total Dosage, the Mean Plasma Concentration (for 4 Days) of Quinacrine and Its Therapeutic Effect in the Standard Blood-induced Therapeutic Trial with McCoy Vivax Malaria*

Total dosage is given rather than priming and daily dosage because of the characteristics of its physiological disposition.

| Patient | Total dosage | Plasma quinacrine, 4-day mean | Therapeutic result | | |
|---|---|---|---|---|---|
| | | | Class I | Class II | Class III |
| | *g.* | *mg./l.* | | | |
| Tra | 0.7 | 44 | | | + |
| Wal | 0.7 | 39 | | | + |
| Bel | 0.7 | 34 | | | + |
| Cra | 0.7 | 32 | | | + |
| Cha | 0.7 | 31 | | | + |
| Dar | 0.85 | 27 | | | + |
| Woi | 0.7 | 26 | | | + |
| Wor | 0.7 | 26 | | | + |
| Qui | 0.7 | 26 | | + | |
| Cas | 0.55 | 25 | | | + |
| Dav | 0.45 | 24 | | + | |
| Far | 0.45 | 23 | | + | |
| Hau | 0.6 | 22 | | + | |
| Mis | 0.6 | 21 | | | + |
| She | 0.45 | 21 | | + | |
| Eic | 0.45 | 21 | | + | |
| Mar | 0.35 | 19 | | + | |
| Chi | 0.45 | 19 | | + | |
| Lor | 0.70 | 19 | | + | |
| Kas | 0.60 | 18 | | + | |
| Sch | 0.70 | 18 | | + | |
| Ami | 0.45 | 16 | | + | |
| Ash | 0.75 | 16 | | + | |
| Ger | 0.55 | 16 | | + | |
| Coo | 0.35 | 15 | | + | |
| Bit | 0.35 | 13 | | + | |
| Dem | 0.35 | 13 | | + | |
| Sca | 0.45 | 12 | | + | |
| Cuo | 0.35 | 10 | | + | |
| McB | 0.45 | 8 | + | | |
| Sau | 0.25 | 7 | + | | |
| Cra | 0.25 | 6 | + | | |
| Kel | 0.25 | 3 | + | | |

in quinine and quinidine, but reasonably high in cinchonine and cinchonidine. By a relatively simple processing, the total alkaloids could be extracted from these barks and used in the construction of a standard product called Totaquine. This was accepted by the U. S. Pharmacopoeia and made available for general use at a time when all quinine was removed from the local market (25, 26, 27, 28) and when the production of quinacrine was too low for any to be available for civilian use.

Quinacrine: The second step which permitted the more effective use of antimalarial agents already available was the result of the acquisition of new information on the physiological disposition and antimalarial activity of quinacrine. It was tentatively assumed, and subsequently established, that, similar to the cinchona alkaloids, the antimalarial activity of quinacrine is a reflection of its concurrent plasma drug concentration (table 7). Consequently, rational usage of the drug required information on those factors which were concerned with the regulation of the plasma quinacrine concentration on any regimen of therapy. It was early demonstrated that, contrary to the cinchona alkaloids, effective plasma quinacrine concentrations are not rapidly achieved on any dosage regimen which administers the same size serially (figure 9). This is because quinacrine is very extensively localized in many tissues of the body and is degraded at a low rate. Accordingly, when used as recommended in 1941, i.e., 0.1 gram three times daily, therapeutically active concentrations are not commonly obtained early in the course of treatment of the clinical attack. It was predictable from this fact that such therapy would not produce an abrupt termination of clinical activity and, in fact, this is what had been found in practice. However, when large priming doses of quinacrine are given during the first 12–16 hours of therapy, high plasma drug concentrations are quickly obtained and clinical activity is promptly terminated (2, 29).

Similarly, it was found that the pattern of weekly dosage has little influence on the plasma quinacrine concentration which is maintained during a course of suppressive therapy. It was also

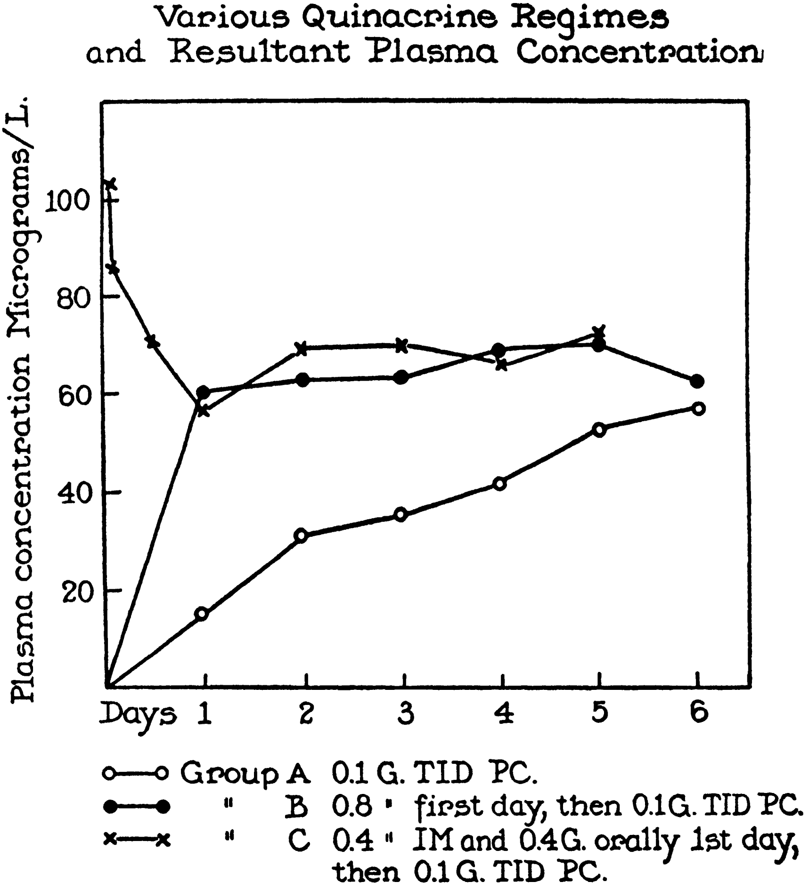

FIG. 9. Plasma quinacrine concentrations achieved with various regimens of therapy. The data given are the means of the observations on some 10 individuals on each regimen. Note the low plasma quinacrine concentrations which obtain during the early days of therapy when quinacrine is administered three times daily on 0.1 gram doses, the high initial plasma quinacrine levels when such a regimen is preceded by 0.8 gram administered during the first day. Not shown in this figure but of considerable importance is the fact that plasma quinacrine concentrations in excess of 150 micrograms per liter are achieved within 15 minutes after the intramuscular administration of 0.4. gram quinacrine dihydrochloride.

found that when the same suppressive dosage is given to a number of normal individuals, there is a very wide variation in the plasma drug concentrations which are achieved and maintained in the individuals of the group, and that when drug administration is limited to 0.4 gram weekly, many individuals in the group attain levels below which one would expect a striking suppressive effect (29, cf. table 7 and figure 10).

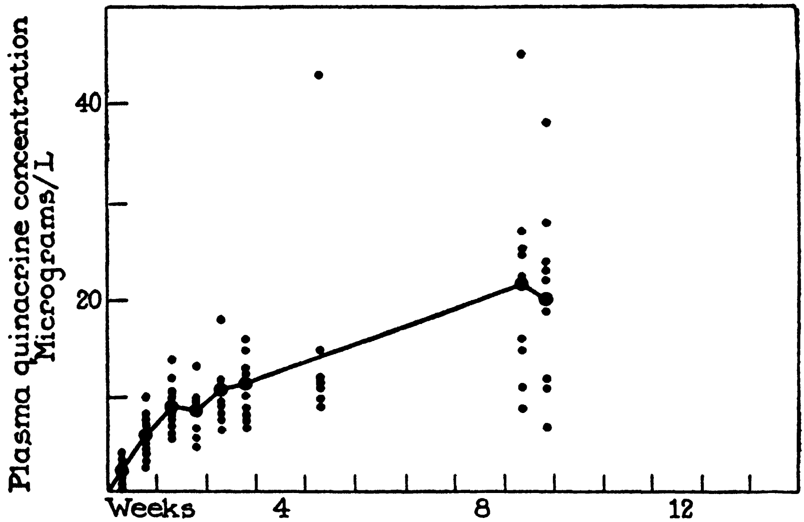

FIG. 10. Plasma quinacrine concentrations observed in a group of normal young adults receiving a total of 0.4 gram of quinacrine dihydrochloride weekly. Regimen of therapy consisted of 50 mg. daily for 6 days, and 100 mg. on the seventh day of each week. More complete data are published elsewhere (29).

It was possible, on the basis of these studies, to recommend a change in the pattern of dosage for quinacrine therapy when used as a suppressive and for the termination of a clinical attack. These recommendations were incorporated in a report to the Committee on Medical Research early in July of 1943 and were shortly adopted by the armed forces (30). One year's experience with what may be termed the rational use of quinacrine was sufficient to demonstrate that the drug is superior to quinine for the routine management of the malarias (31). Quinacrine will prevent the inception of clinical falciparum malaria when given as a suppressive, and effect a prompt and definitive cure when the infection is once established. However, quinacrine will not prevent the inception of vivax malaria although it is highly effective in suppressing its clinical manifestations and it will not effect a definitive cure when the infection is once established. It was not known in 1943 whether or not the latter two limitations were fundamental in character. However, the effectiveness of routine quinacrine therapy was such that in the summer of 1943 it seemed likely the program could focus more of its attention upon the development of curative agents for vivax malaria. This marked the end of the first period of studies and the beginning of the second.

*Search for New Drugs.* The major effort of the next phases of the malaria program was devoted to the development of curative agents in vivax malaria. This was a cooperative effort, in a true sense, and involved the participation of many synthetic chemists, pharmacologists and clinicians, whose activities were correlated by the Board for the Coordination of Malarial Studies. The results of these studies will undoubtedly be the subject of many presentations by contributing laboratories in the near future and need only be summarized in a brief manner here (cf. 32).

It was believed that the development of an antimalarial with a high degree of curative action in vivax malaria could be approached with reason by more than a single way. One approach could be based upon an hypothesis which held the fundamental metabolic organization of the persisting tissue forms of *P. vivax* to be essentially the same as that of the erythrocytic forms of the plasmodium and that suppressive and curative activities in vivax malaria could be the same. Due to a difference in its cellular environment, the tissue form was assumed to be less susceptible to the antimalarial effect of drugs such as quinacrine. Accordingly, a reasonable approach to the development of curative agents appeared to lie in the direction of obtaining more active drugs as evidenced by their ability to exert an action upon the erythrocytic forms of the parasite, i.e., suppressive activity as manifested in the blood-induced infection. It was hoped that if the intensity of this type of antimalarial activity was sufficiently great in the case of any drug, then it would not only interrupt the erythrocytic phase of the vivax parasites, but would also exert a curative action and obliterate the persisting tissue phase of a naturally acquired infection and so cure the disease.

Another approach could be based upon an hypothesis which held the fundamental metabolic organization of the persisting tissue forms of *P. vivax* to be different from that of the erythrocytic forms of the plasmodium, at least insofar as the susceptibilities of their essential biological systems are concerned. Accordingly, a chemotherapeutic agent might affect the tissue forms through an action which is qualitatively different from any which produces a dramatic effect upon the asexual forms in the erythrocyte, i.e., the blood-induced infection has little value in the search for curative agents. In accordance with this hypothesis, it was quite possible to miss a curative agent unless a number of representatives of each group of chemicals studied were examined for curative action. Compounds could then be selected for this type of activity because of special activities other than suppressive in the avian infections, or, they could be screened for curative action in the human vivax infection without prior experimental trial.

It was generally agreed, in the fall and winter of 1943, that sufficient evidence was not available for one to decide which of these two hypotheses was more reasonable. Consequently, there was considerable discussion as to whether blood-induced infections could be of value in a program, the end of which was the development of curative agents for vivax malaria. However, this type of infection was continued in use on a rather extensive scale, its use being based on the tentative acceptance of the reasonableness of the first working hypothesis. As a logical consequence and as the major effort at that time, a systematic attempt was made to increase antimalarial activity in a number of the chemical series then under exploration and the best representative in each series was selected on the basis of information from blood-induced infections, and examined for curative action in mosquito-induced vivax malaria. Actually, the overall procedure adopted represented a partial compromise between the two working hypotheses. Certain of the compounds studied for curative action had relatively little suppressive action, their selection being based upon two considerations. They were representatives of chemical series as yet untried for curative action and although they might have had little suppressive activity, the compound tested was better in this respect than the other members of the series examined. In addition, any compound showing a special type of activity in the avian infections, such as curative or prophylactic, was also tried for curative action in vivax malaria.

The advantages of this approach, at that stage of the program, were three. First, it was believed likely, with the leads then available, that suppressive antimalarial activity could be increased manyfold in several different types of compounds and, as the result of this effort, compounds would shortly become available with which to test the correctness of the first working hypothesis. Second, it would permit the study of a number of chemical series, as yet unexamined, for their possession of curative action and perhaps establish a correlation between special activities in avian infections and curative activity in vivax malaria. Third, it seemed reasonable to suppose that this approach to the problem would result in the development of antimalarials superior to quinacrine although they might have the same fundamental limitations. The third possibility was important. It was desirable to have available antimalarials other than quinacrine, should the long-term continuous administration of quinacrine to the human be accompanied by toxic manifestations which at the time could not be predicted.

It was early demonstrated beyond doubt that the suppressive antimalarial activity of a compound, when measured in a single avian infection, may have little prediction value for the situation obtaining in the suppression of peripheral parasitemia in the human malarias.[9] It was later demonstrated that the sum total of the information, when derived from the study of the activity of a compound or series of compounds in several avian infections using several avian hosts, does have fair prediction value in the selection of compounds for trial as suppressives in the human malarias. Lastly, it was demonstrated, within the compounds studied, that none had higher antimalarial activity of a suppressive nature in both human infections than had been observed in at least one of the avian infections.[10] This information was accumulated incidental to the survey of a very large number of compounds (ca. 12,000) for activity in the avian infections, the survey of a limited number of compounds (ca. 65) for suppressive activity in the human infections and a selected number of the latter group (ca. 20) for prophylactic and/or curative action in vivax malaria (32).

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[9] The data available on antimalarials at the beginning of the program and that from allied fields of chemotherapy led Dr. E. K. Marshall, Jr., Chairman of the Pharmacology Panel, to this conclusion.

Out of these extensive studies no compounds were developed with prospects of being useful as curative agents in vivax malaria although several have unquestioned advantages over quinacrine and quinine. For example, a plasma quinine concentration of 5 mg. per liter maintained for 4 days terminates a blood-induced infection or a clinical attack of a mosquito-induced infection due to the McCoy strain of *P. vivax.* An equivalent effect will be produced by 30 micrograms per liter of quinacrine. However, plasma concentrations of quinine in excess of 12 mg. per liter and of quinacrine in excess of 150 micrograms per liter are not generally well tolerated.

Of the newer compounds developed during the fall and winter of 1944–1945, there is one (SN–7618)[11] which is rather well tolerated at dosage schedules which produce plasma drug concentrations some 30 times those required to terminate the clinical attack (21). Others, whose activities have been placed less precisely in terms of plasma drug concentrations, can be administered in daily doses many times those required to produce a demonstrable antimalarial effect. Nevertheless, these agents have nothing to offer as curative agents in vivax malaria. It may be concluded from these observations that the major working hypothesis selected for trial in 1943 in the attempt to develop curative agents in vivax malaria is not correct.

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[10] The substantiation of these general concepts was as the result of the combined efforts of all Scientific Research and Development contractors on both the pharmacological and clinical levels. The experimental facts themselves will be contained in a monograph entitled, "A Survey of Antimalarials, 1941–1946," edited by Dr. F. Wiselogle and prepared by the Office for the Survey of Antimalarial Drugs. This should be available by the fall of 1946.

[11] *7-chloro-4-(4-diethylamino-1-methylbutylamino)quinoline.*

Before considering the third plase of the studies it will be of some interest to take note of the potentialities of certain of the agents which, at least to some extent, are by-products of an unsuccessful attempt to produce a curative agent for vivax malaria. Among the more promising compounds are some which may be expected to effect complete suppression when administered once weekly in a well-tolerated dose. They will also effect an abrupt termination of a clinical attack of vivax and a cure of falciparum malaria when administration is limited to one or, at most, two days. None of these highly effective agents has, as yet been fully exploited. However, information is at hand which permits the prediction that they will constitute a relatively simple means for the complete control of malaria in many areas due to the lessening of the administration problem of suppressive therapy as compared to quinacrine. They may also, in specific areas, contribute to the eradication of the malaria through their ability to curtail transmission of the disease. Exploration of the advantages to be derived from the use of some of these newer agents is now under way.

Of importance to the attainment of one of the ultimate objectives of the program was the conclusion that a simple increase in antimalarial activity as evidenced by an effect against the erythrocytic forms of the parasite cannot, *per se*, be expected to lead to curative drugs for vivax malaria. The obtaining of this information marked the beginning of the present stage of the malaria studies. This has been characterized by the direct approach to the problem of devising curative agents which are now assumed to require qualitatively different actions from suppressive drugs whose activities are reflected in a reduction of peripheral parasitemia in vivax and falciparum malaria. These studies are proceeding in several laboratories and with some prospect of success.

A serious obstacle to success in this endeavor stems from the fact that, with our present knowledge, it is not possible to use the experimental avian malarias effectively to screen compounds prior to their selection for trial as curative agents in vivax malaria. Drugs have been developed which possess prophylactic and/or curative action in one or another of the avian infections but, generally speaking, these actions are not a reflection of a similar action in vivax malaria. Actually, there is, as yet, no general correlation between these special actions in the avian infections and comparable action in the human infections (32). The promise that curative drugs will eventually be found stems solely from the recent confirmation of the earlier studies on the curative action of pamaquine, in 8-aminoquinoline.

It seems reasonably certain that the older investigations on the antimalarial activity of pamaquine led to conclusions which are essentially correct (33). That is, pamaquine, when administered at high dosage, has a curative action in vivax malaria when administered concurrently with quinine over a long period of time. This is a fact of importance. It demonstrates that the persisting tissue forms of the plasmodia which are held to be responsible for the relapse in vivax malaria are subject to the lethal action of a drug to which the type of host cell within which they reside is not also generally susceptible. Furthermore, the curative action of pamaquine makes available a specific lead toward the synthesis of better tolerated curative agents.

This lead is now being explored extensively. It did not receive attention earlier in the program for three reasons. It was known that pamaquine analogs had received systematic study by the Germans, French, and the Russians, both before and after the development of pamaquine, and no better drug had been announced. It was also known that pamaquine and many of its analogs possess seriously toxic effects when administered at a dosage well below that which is generally curative. Finally, it was hoped that a curative agent might be found in other series of substances which were characterized by a lesser toxicity. It was not until the last possibility seemed unlikely, at least in the near future, that it was deemed advisable to embark upon an extensive study of 8-aminoquinolines.

It is now known that previous exploration of the 8-aminoquinolines was inadequate to be certain that pamaquine is the best drug to be derived from this series. Furthermore, the careful study of the antimalarial activity and toxicity of pamaquine and a selected series of 8-aminoquinolines seems to indicate that such an exploration will be profitable. A consideration of what has been done with other chemical series, whereby one or another aspect of antimalarial activity has been greatly increased without a comparable increase in toxicity, makes the prospect of obtaining a useful agent from this group of substances rather bright.

*The Detailed Study of Cinchonine.* The space remaining will be spent in a brief consideration of certain experimental data which relate to the antimalarial activity of the cinchona alkaloids and which are deemed to be essential before an attempt can be made at an examination of the discrete mechanisms involved in the production of their antimalarial effects. These studies may be taken as the prototypes of others which must be undertaken before the biological mechanisms which underlie any specific antimalarial activity can be approached in a rational manner.

Earlier speculations concerning the mechanism of the antimalarial actions of the cinchona alkaloids included the possibility that these compounds have no direct action on the plasmodia. Similar suggestions have been made concerning the action of some of the antibacterials but these have been found to be without basis when subjected to direct experimental examination. It seemed probable that a similar situation would obtain with the cinchona alkaloids, and that their antimalarial activity is derived from an ability to interfere with some biological system within the parasitized erythrocyte which is essential for the growth and multiplication of the parasite.

An inquiry into the details of such an effect presupposes knowledge of the molecular species of at least one of the cinchona alkaloids which is capable of such an action. This need for information is highlighted by an appreciation of the marked difference between the antimalarial activities of the four alkaloids when based on the plasma drug concentrations as opposed to the oral dosages which are required to produce any given therapeutic effect in the standard blood-induced infection (table 8). It seemed quite possible, in view of this circumstance, that, incidental to the more or less complete metabolic change of each of these structurally similar compounds, an intermediate substance is formed from each of the alkaloids with common chemical characteristics and this substance is the active antimalarial. In such a situation, the amount of active material formed each 24 hours might be related more to the oral dosage of the alkaloid than to the plasma alkaloid concentrations attained.

TABLE 8

*Plasma Drug Levels of the Cinchona Alkaloids Necessary to Obtain Class III Effects in Standard Blood-induced Infection and the Plasma Drug Levels Achieved on Serial Oral Dosage of Each Alkaloid*

The data on quinidine plasma drug concentrations during the serial administration of 1.0 gram of drug daily were obtained on only three patients. However, from other information the value determined would appear to be in the proper order of magnitude.

| | Mean plasma concentration of alkaloid (mg./l.) | | | |
|---|---|---|---|---|
| | Quinine | Quinidine | Cinchonine | Cinchonidine |
| Class III effect . | 5.0 | 1.0 | 0.1 | 3.0 |
| 1.0 gram daily . | 5.6 | (4.6) | 0.2 | 2.4 |
| Ratio $\frac{\text{1.0 gram daily}}{\text{Class III effect}}$ | 1.1 | (4.6) | 2.0 | 0.8 |

Opposed to the acceptance of this hypothesis was the reasonably good correlation with each alkaloid between the plasma drug concentrations of each alkaloid and their antimalarial effects and, in any given series of observations on a single alkaloid, the lack of a similarly good correlation between oral dosage and antimalarial effects (figure 11). However, the estimated difference between the antimalarial activities of quinine and cinchonine, when based on the plasma alkaloid concentrations, is so great, that a search was made for more definitive evidence concerning the possible role of degradation in conditioning the antimalarial activity

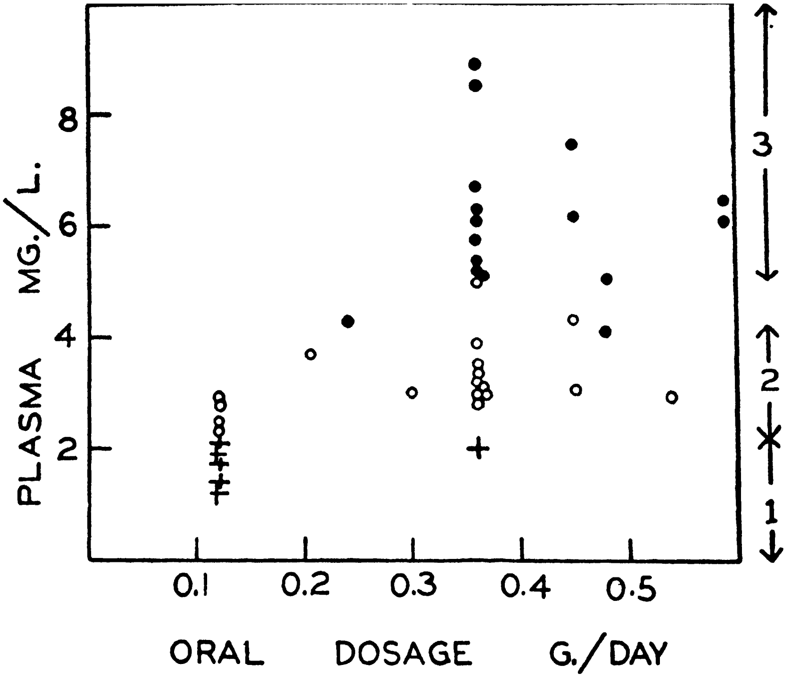

FIG. 11. The relationship between the oral administration of quinine and the plasma quinine concentration observed in a series of therapeutic trials with both blood-induced and mosquito-induced McCoy vivax malaria. Each symbol represents the mean plasma quinine concentration obtaining during a four-day course of therapy. Quinine was administered as the hydrochloride in gelatine capsules at four- or six-hour intervals. The therapeutic effect in each test is indicated by the symbol used. Dots represent therapeutic tests which resulted in Class III effects; circles, Class II effects; crosses, Class I effects.

of these compounds. Generally speaking, a metabolic change may influence the therapeutic activity of an agent in one of three ways. It may produce an active agent from an inert substance, as contained in the above hypothesis covering the cinchona alkaloids and as appears to be the case in the "prontosil soluble"—sulfanilamide system; it may limit the action of a substance through changes of chemical structure which minimize or remove the ability of a substance to exert an action; or, a substance may exert an action insofar as it enters a biological system and exerts an effect incidental to the operation of the system which results in its metabolic change.

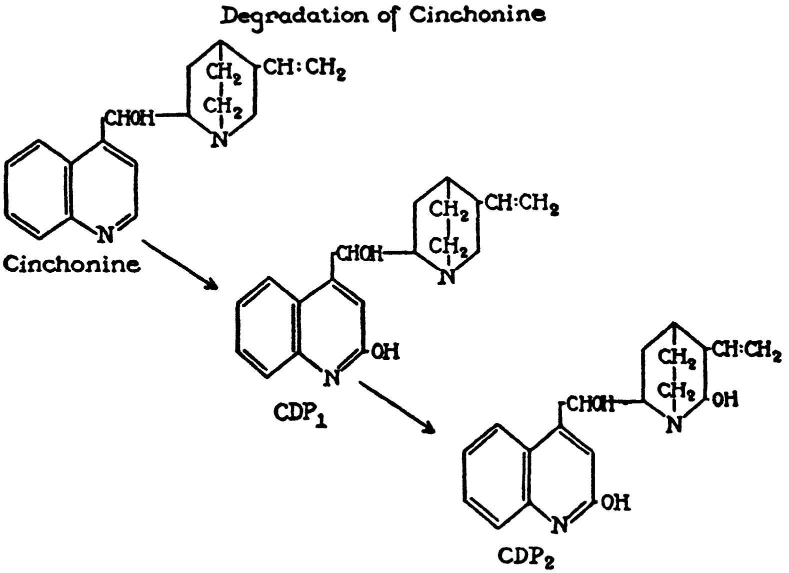

FIG. 12. Proposed scheme for the main route of metabolism of cinchonine in the human.

Cinchonine was selected for initial study because its coefficient of metabolism is much higher than that of the other cinchona alkaloids and because, in contrast to quinine and quinidine, it has a simpler chemical structure. Dr. Joseph Koepfli, California Institute of Technology, and Dr. Lyman Craig of the Rockefeller

Institute for Medical Research have been most helpful in these studies through their identifications of the metabolic products which were isolated.

The main route of degradation of cinchonine in the human appears to involve two serial steps (figure 12): the first of these is an addition of oxygen to the quinoline nucleus with the formation of a carbostyril; the second, an addition of oxygen to the quinuclidine nucleus, perhaps at the ring carbon adjacent to the nitrogen.

The chemical basis for these deductions need not be given. However, the evidence for accepting these two serial oxidations as the main channel of metabolism will be reviewed. This was obtained in experiments wherein the urinary excretion of isolatable material was studied in the human during the administration of cinchonine or its carbostyril. The data summarized in table 9 indicate that in excess of 80 per cent of orally administered cinchonine is recoverable in the urine as cinchonine or its first or second metabolic product. Similar experiments with the first metabolic product are also contained in table 9. It was only

TABLE 9

*The Metabolic Fate of Cinchonine*

Recovery of cinchonine and its metabolic products from the urine of 3 subjects given 3 different serial oral regimens of cinchonine.

| Mean % of daily dose recovered per 24 hours | | | |
|---|---|---|---|
| Cinchonine | $CDP_1$ | $CDP_2$ | Total |
| 4 | 55 | 22 | 81 |

Recovery of $CDP_1$ and $CDP_2$ following a single oral dose of 2.0 grams $CDP_1$ to a single individual.

| % of dose recovered in 48-hour urine | | | |
|---|---|---|---|
| Cinchonine | $CDP_1$ | $CDP_2$ | Total |
| 0 | 30 | 14 | 44 |

possible in these experiments, performed upon different subjects, to account for some 40 per cent of the administered material as the first or second degradation product. However, these findings are in keeping with the view that the first reaction (figure 12) is more complete and proceeds at a somewhat more rapid rate than the second, and that small quantities of other metabolic derivatives are normally formed when cinchonine is administered. However, the data indicates that these are derived largely, if not exclusively, from the first or second metabolic product rather than directly from cinchonine itself. The final quantitation of this system must await further studies of these general types performed serially upon individual subjects.

The oxidation of cinchonine to a carbostyril is due to the operation of an enzyme system (34) contained in liver and other tissue homogenates which have been shown by Kelsey to produce a carbostyril from quinine (35). The enzyme system has not been isolated from human tissues although the product of its action, i.e., the carbostyril, has been, as noted above, isolated and identified in the urine of humans receiving cinchonine. The enzyme has been isolated for study from rabbit liver. Studies on the kinetics of its action in simple chemical systems oppose the view that any stable intermediate substance is formed between cinchonine and its carbostyril except incidental to the combination of cinchonine and enzyme during the catalyzed oxidation (34). It seems likely, then, that the antimalarial activity of cinchonine is due to one or another of these three molecular species or to a combination of the activities of all three. With this thought in mind, cinchonine was administered to patients so as to permit the isolation and purification of the first and second metabolic products, the former in sufficient quantity to study its antimalarial activity in blood-induced vivax malaria.

Table 10 summarizes the data of experiments which examine the antimalarial activity of cinchonine in the standard blood-induced infection. These data can serve as a primary standard of reference for the examination of the antimalarial activity of the first metabolic product. Daily doses in the order of 0.5–1.0

TABLE 10

*The Relationship Between Dosage, the Mean Plasma Level[12] of Cinchonine (4 Days) and Its Therapeutic Effect in the Standard Blood-induced Therapeutic Test with McCoy Vivax Malaria*

| Patient | Daily dosage | Mean plasma conc. | Result | | |
|---|---|---|---|---|---|
| | | | Class I | Class II | Class III |
| | *g.* | *mg./l.* | | | |
| SP | 1.0 | 0.5 | | | + |
| ER | 2.0 | 0.4 | | | + |
| KO | 1.0 | 0.4 | | | + |
| CA | 0.75 | 0.3 | | | + |
| JO | 1.0 | 0.1 | | | + |
| MA | 1.0 | 0.1 | | | + |
| DA | 0.5 | 0.07 | | + | |
| TU | 0.5 | 0.05 | | + | |
| AN | 0.5 | 0.05 | | + | |
| CO | 0.5 | | | + | |
| RE | 0.5 | | | + | |
| NI | 0.5 | | | + | |

gram produce plasma cinchonine concentrations no greater than 0.1 mg. per liter[12] and are accompanied by Class III effects. Table 11 summarizes the data obtained in a similar series of therapeutic trials performed with the first cinchonine metabolic product. Much higher concentrations of this product are found in the plasma during its oral administration, as compared with cinchonine, but these produce lesser antimalarial effects. A minimum of 3 grams daily is required to produce a Class III

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[12] Truly pure cinchonine, i.e., homogeneous, is not available in sufficient quantities for experiments of this general type. Most "pure" cinchonine appears to be contaminated to a variable extent by quinine and dihydrocinchonine. Spectrophotometric and fluorometric studies of the material analyzed as cinchonine by the method used (36) indicate that something less than fifty per cent of the apparent plasma cinchonine concentrations at the lower plasma alkaloid concentrations is due to quinine. These large proportions are attributable to a minor contamination of the cinchonine used by quinine and a coefficient of metabolism for cinchonine which is some 50 to 100 times greater than that of quinine.

TABLE 11

*The Relationship Between Dosage, the Mean Plasma Level (4 Days) of the First Metabolic Product of Cinchonine and Its Therapeutic Effect in the Standard Blood-induced Therapeutic Test with McCoy Vivax Malaria*

| Patient | Daily dosage | Mean plasma conc. | Result | | |
|---|---|---|---|---|---|
| | | | Class I | Class II | Class III |
| | *g.* | *mg./l.* | | | |
| ST | 3.0 | 3.4 | | | + |
| BR | 3.0 | 2.4 | | + | |
| OR | 1.5 | 1.3 | | + | |
| BU | 0.75 | 1.1 | + | | |
| MU | 0.75 | 0.7 | + | | |

effect in the standard infection and this only when accompanied by plasma drug concentrations in the order of 3 or more mg. per liter.

Information which permits an integration of these two sets of data was obtained in observations wherein cinchonine was administered to patients in different daily dosages (table 12). These data demonstrate that significant concentrations of the first degradation product are always present during the administration of cinchonine. However, it may be estimated that, at the dosage of cinchonine required to produce a Class III effect, the

TABLE 12

*Plasma Levels of Cinchonine and Cinchonine Degradation Product, Achieved on Serial Oral Dosage of Cinchonine (3 Subjects)*

| Daily dosage | Mean Plasma drug levels | |
|---|---|---|
| | Cinchonine | C. degradation product |
| *g.* | *mg./l.* | *mg./l.* |
| 0.5 | 0.05 | 0.8 |
| 1.0 | 0.3 | 1.1 |
| 2.0 | 0.8 | 3.1 |

first metabolic product contributes at best a fourth of the total antimalarial activity.

The further analysis of the system requires a consideration of the possible antimalarial activity of the third species in the cinchonine system, i.e., the second metabolic product. However, since the latter is formed from the further oxidation of the first product, the antimalarial activity observed during the administration of the carbostyril is due to the combined effect of both products. It follows, then, from the examination of the activity of the first degradation product, that metabolic derivatives of this are relatively unimportant in conditioning the overall antimalarial activity of cinchonine.

It appears likely, then, that the antimalarial activity of cinchonine and, by analogy, that of the other cinchona alkaloids, is derived predominately from the ability of each of the drugs, rather than an active intermediate, to participate in an essential biological system of the parasite or a shared biological system in the parasitized erythrocyte. This conclusion may serve as the basis for further speculations concerning their mechanism of action. It is possible that the operations of chance have produced a situation whereby a given therapeutic effect requires roughly equivalent oral doses of each but is produced by widely different plasma drug concentrations. It is also possible that the ability of each alkaloid to participate in the biological system within the host which results in its degradation is a simple reflection of the chemical characteristics of the alkaloid which condition its ability to participate in an analogous biological system within the parasite which results in the production of an antimalarial effect. The mechanisms underlying the antimalarial activity of cinchonine and the other cinchona alkaloids warrants further and more detailed study. This should be facilitated by the recent finding that the antimalarial activities of the conchona alkaloids and certain other antimalarials can be studied in cultures of growing erythrocytic forms of the parasites (37, 38). The plasma concentrations of several antimalarials required to exert an inhibitory action in these cultures are of the same order as those which are required to produce Class III effects in the human host (38).

It is to be emphasized that the importance of the observations that have been reported on the antimalarial activity of the cinchona alkaloids no longer stems from considerations of their use in the suppression and treatment of malaria. Rather, they have importance in that they define the general type of background information which must be obtained before an approach may be made towards an examination of the discrete biological systems within the parasite or parasitized erythrocyte which are concerned with the antimalarial actions observed.

## BIBLIOGRAPHY

1. Symposium on Human Malaria, Washington, A. A. A. S., 1941, Publ. #15.
2. Shannon, J. A., and Earle, D. P., Jr., *Bull. N. Y. Acad. Med.*, 1945, 21, 467.
3. Shannon, J. A., *Bull. N. Y. Acad. Med.*, 1946, 22, 345.
4. James, S. P., *Trans. Roy. Soc. Trop. Med. Hyg.*, 1931, 24, 477.
5. Boyd, M. F., Stratman-Thomas, W. K., *Amer. J. Hyg.*, 1934, 20, 488.
6. Raffaele, G., *Riv. Malariol.*, 1937, 16, 185.
7. Ciuca, M., Ballif, L., Chelaresev, M., Isanos, M., and Glaser, L., *Riv. Malariol.*, 1937, 16, 85.
8. Boyd, M. F., and Matthews, C. B., *Amer. J. Trop. Med.*, 1939, 19, 69.
9. Boyd, M. F., A. A. A. S., 1941, Publ. #15, 163.
10. Huff, C. G., and Coulston, F., *J. Infect. Dis.*, 1944, 75, 231.
11. Coatney, G. R., Ruhe, D. S., Cooper, W. C., Josephson, E. S., Young, M. D., and Burgess, B. E., personal communication.
12. Fairley, M. H., *Trans. Roy. Soc. Trop. Med. Hyg.*, 1945, 38, 311.
13. Boyd, M. F., Kitchen, S. F., and Muench, H., *Amer. J. Trop. Med.*, 1936, 16, 583.
14. Staff of the Research Service, Third (NYU) Medical Division, Goldwater Memorial Hospital, unpublished observations.
15. Boyd, M. F., *Amer. J. Trop. Med.*, 1940, 20, 269.
16. Ehrman, F. C., Ellis, J. M., and Young, M. D., *Science*, 1945, 101, 377.
17. Brodie, B. B., and Udenfriend, S., *J. Pharmacol. and Exp. Therap.*, 1943, 78, 154.
18. Fourth General Report of the Malarial Commission, The Treatment of Malaria, *Bull. of the Health Organization, League of Nations*, 1937, 6.
19. Coatney, G. R., and associates, unpublished observations.
20. Ferrebee, J. W., and Geiman, Q. M., J. Infect. Dis., 1946, 78, 173.
21. Board for the Coordination of Malarial Studies, War Time Research in Malaria, *Science*, 1946, 103, 8.

22. Berliner, R. W., Bigelow, F. S., and Kennedy, T. J., Jr., Fed. Proc., 1946, 5, 165.
23. Shannon, J. A., *Ann. N. Y. Acad. Sci.*, 1943, 44, 455.
24. Fletcher, W., Notes on the Treatment of Malaria with the Alkaloids of Cinchona, London, John Bale, Sons and Dannielsson, Ltd., 1923.
25. Editorial: Restrict Sale of Quinine, *J.A.M.A.*, 1942, 119, 1512.
26. Weed, L. H., *J.A.M.A.*, 1942, 120, 1043.
27. The Therapeutic Efficiency of Totaquine in Human Malaria, *Quart. Bull. Health Organization, League of Nations*, 1934, 3, 325.
28. Dawson, W. T., *Internat. Clin.*, 1930, 2, 121.
29. Shannon, J. A., Earle, D. P., Jr., Brodie, B. B., Taggart, J. V., and Berliner, R. W., *J. Pharmacol. and Exp. Therap.*, 1944, 81, 307.
30. U. S. War Department, Office of the Surgeon General. The Drug Treatment of Malaria, Suppressive and Clinical, Circular Letter No. 153, *J.A.M.A.*, 1943, 123, 205.
31. Board for the Coordination of Malarial Studies, National Research Council, Quinacrine Hydrochloride (Atabrine) for Malaria, *J.A.M.A.*, 1944, 125, 977.
32. Wiselogle, F., Editor, A Survey of Antimalarials 1941–1946, Edwards Bro., Ann Arbor, Mich., 1946, in preparation.
33. Sinton, J. A., Smith, S., and Pottinger, D., *Ind. J. Med. Res.*, 1929–30, 17, 793.
34. Knox, W. E., J. Biol. Chem., 1946, 163, 699.
35. Kelsey, F. E., Geiling, E. M. K., Oldham, F. K., and Dearborn, E. H., *J. Pharmacol. and Exp. Therap.*, 1944, 80, 391.
36. Brodie, B. B., Udenfriend, S., and Dill, W., Fed. Proc., 1946, 5, 126.
37. Ball, E. G., Anfinsen, C. B., Geiman, Q. M., McKee, R. W., and Ormsbee, P. A., *Science*, 1945, 101, 542.
38. Berliner, B. W., Fed. Proc., 1946, 5, 164.

# PHYSIOPATHOLOGY AND SURGICAL TREATMENT OF CONGENITAL CARDIOVASCULAR DEFECTS[1,2]

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THE fame of William Harvey is of the enduring type that increases with the passing of years. It was my privilege to be present in London in 1928 at the tercentennial celebration of the discovery of "the movements of the heart and blood." Undoubtedly others in this audience were equally fortunate and departed, as did I, with an even more profound respect for the great anatomist and physiologist of the seventeenth century. The objectives of the Harvey Society are not limited to the diffusion of scientific knowledge in selected subjects in anatomy and physiology but are extended to include pathology, bacteriology, pharmacology, and physiological and pathological chemistry—to the alterations which take place as the result of both structural abnormalities and disease. In this Harvey Lecture it gives me a sense of gratification to discuss certain abnormalities of the circulation, the normal physiology of which was first established by Harvey himself, and to outline the recent advances which have been made in the surgical treatment of these conditions.

Congenital cardiovascular defects are not rare in infancy, and it is only because of the high early mortality rate that the incidence after the age of 12 years is low. The complex nature of the defects, which adds to the difficulty of diagnosis, particularly in infants, and the lack of successful therapeutic methods resulted for years in a general neglect of this field. It is only in recent years that greater interest has been aroused as a result of

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[1] Lecture delivered November 15, 1945.

[2] From the Department of Surgery of The Johns Hopkins University and Hospital.

the work of Maude Abbott, Taussig, Gross, Burwell and others. Their investigations have shown that it is no longer adequate simply to differentiate congenital from acquired heart disease. In a large percentage of cases one should be able to diagnose the particular defects which are present. Advances in surgical therapy for certain types make it all the more important that these types be recognized. That the advances are recent is shown by the statement in the 1937 edition of "Heart Disease" by White (1) to the effect that "there is no curative treatment, surgical or medical, for congenital cardiac defects."

Maude Abbott (2) devised a clinical classification of congenital heart disease in which the patients are divided into three groups. The first group includes those without abnormal communications or shunts between the right and left sides of the heart. Cyanosis is not a part of the picture. This group includes patients with simple dextrocardia, anomalies of the pericardium, primary congenital hypertrophy of the heart, pure subaortic or aortic stenosis, pure mitral stenosis, and coarctation of the aorta. The second group embraces patients with arteriovenous shunt in whom arterial blood enters the pulmonary circulation. Cyanosis is usually not observed. There is, however, possible terminal or transient reversal of flow with cyanosis due to the entrance of venous blood into the systemic circulation. Patients in this second group have defects of the interauricular septum, defects of the interventricular septum, localized defects of the aortic septum, and patent ductus arteriosus. This is the potentially but rarely cyanotic group. The third group according to Abbott includes those patients in whom cyanosis associated with the entrance of venous blood in large quantities into the systemic circulation is a prominent feature. Included among the many causes are defects of the interventricular septum with dextroposition of the aorta, tricuspid stenosis, tricuspid atresia with septal defects, transposition of arterial trunks with defects of the ventricular septum, persistent truncus arteriosus, and the tetralogy of Fallot.

It happens that advances in surgical therapy have been made in a representative of each of the three groups described by Abbott—coarctation of the aorta in the acyanotic group, patent ductus arteriosus in the usually acyanotic but occasionally cyanotic group, and pulmonic stenosis (usually the tetralogy of Fallot) in the cyanotic group. This lecture will consist in the main of a consideration of patent ductus arteriosus, of coarctation of the aorta, and of the tetralogy of Fallot with particular emphasis on the latter condition.

## PATENT DUCTUS ARTERIOSUS

Although a procedure for closure of patent ductus arteriosus had been described by Munro (3) in 1907 and although the operation had been attempted by Strieder (4), the first successful closure of an open ductus was performed by Gross (5) in 1938. According to Abbott, patent ductus arteriosus occurs together with other congenital heart lesions twice as often as it is observed as a single abnormality. The impression that there is usually an associated lesion probably accounts for the years that elapsed between the initial description of the operative procedure and the successful undertaking by Gross. As regards the incidence of associated lesions it is likely that Abbott's statistics are misleading in that the figures were derived from autopsy material and probably represented an undue number of the more complicated conditions. Studies in the past six years have shown that patent ductus arteriosus frequently exists as an isolated lesion. It is estimated by Keys and Shapiro (6) that there are approximately 20,000 persons in this country with a patent ductus. Great credit is due Gross and Hubbard not only for the benefit accorded patients with patent ductus arteriosus but for the stimulus to further exploration of the problems of congenital heart disease. Gross originally considered bacterial endarteritis a contraindication to operation, and it is due to the work of Touroff (7) that this view has been altered.

The most extensive studies of the effects of patent ductus arteriosus on the circulation have been carried out by Eppinger, Burwell and Gross (8). These workers demonstrated that the blood flowed from the aorta to the pulmonary artery and not in the reverse direction, that the volume of the leak in patients with large openings constituted 45 to 75 per cent of the blood expelled by the left ventricle, and that because this large volume of blood returned to the left ventricle without passing through the right, the output of the left ventricle was two to four times that of the right. Keys (9) found that the leak may constitute from 20 to 70 per cent of the left ventricular output. Even though there are reasons for doubting the magnitude of the recorded alterations, it appears that the overworking of the left ventricle probably explains the primary occurrence of left ventricular failure when failure occurs.

The recorded studies on the circulation explain and support the clinical observation that patients with failure or diminished reserve are benefited by closure of an open ductus. They also indicate that closure of the ductus in the absence of signs of failure may protect the heart from an eventually disabling burden. It must be borne in mind, however, that many persons with a patent ductus never have any disability as a result of the abnormality. There is a difference of opinion whether operation is indicated for patients without symptoms. Advances in the chemotherapy of bacterial endarteritis make less urgent the necessity for ligating the ductus from a prophylactic viewpoint. It does appear, however, that closure of the ductus is indicated for a patient with an established infection whether it does or does not show a favorable response to the use of chemotherapeutic agents.

I shall speak briefly regarding methods for effecting permanent closure of patent ductus arteriosus. The first method used by Gross was that of simple ligation of the ductus. It is known that the lumen of a large artery which is closed by ligation in continuity may become patent again as the ligature cuts through the wall. It is not surprising that Gross abandoned this method after re-establishment of the fistula occurred in several of his first 14 patients. The second method employed consisted of the use of cellophane in addition to the ligature. Despite the fact that cellophane produces scarring, the ductus reopened in several of the patients. The third method used by Gross (10) consisted of complete division of the ductus and closure of the two ends. During the past three years my associates and I have operated upon 19 patients with patent ductus arteriosus, most of whom were referred by Dr. Helen Taussig. Various methods, including division and closure of the ductus, have been used. There has been only one patient in whom there was evidence that the lumen became re-established; in this case a simple ligature had been used. Although no unusual difficulties have been encountered, I think that division and closure of the ductus is an unnecessarily dangerous operation. The method which we employ at the present time consists of (1) the careful exposure of the entire length of the ductus, (2) the use of a purse string suture ligature of silk at the aortic and pulmonic ends of the ductus, (3) the use of two through and through mattress sutures of silk between the purse string ligatures, and (4) the placing of a ligature of umbilical tape over the mattress sutures. There has been no evidence of reopening of the ductus in the seven cases in which this method has been used.

As I have stated previously, successful surgical treatment of patent ductus arteriosus has led to an increased interest in all types of congenital heart disease. My interest, however, in the next disorder to be discussed, namely coarctation of the aorta, was aroused as a result of experiments on another subject and a chance conversation with Dr. E. A. Park. In an attempt to produce pulmonary arteriosclerosis, Levy and I (11) in 1938 performed operations on dogs in which the proximal end of the severed left subclavian artery was anastomosed to the distal divided end of the left pulmonary artery. In other words, the systemic and pulmonary circulations were connected by a suture anastomosis. Some of these animals were followed for as long as six years after operation. Much to our disappointment, arteriosclerosis did not develop. The blood pressure in the pulmonary artery only a short distance beyond the point of anastomosis was less than half that in the systemic arteries. Except in instances in which partial thrombosis occurred at the anastomotic site, microscopic examination showed no noteworthy alterations in either the left pulmonary artery or the lung. During a discussion of these results with Dr. Park, he suggested that the principle of altering the course and function of a vessel might be used in the treatment of coarctation of the aorta.

### COARCTATION OF THE AORTA

Coarctation of the aorta is a narrowing of the lumen of the vessel of varying degrees in the vicinity of the insertion of the ductus arteriosus. It was found in 142 of Abbott's series of 1,000 cases of congenital cardiovascular defects. It was the primary lesion in 79 of these and was associated with other abnormalities in 63 cases. The commonest form of aortic coarctation is the so-called adult type in which is a localized constriction of the aorta usually at or just below the insertion of the ductus arteriosus. Less frequent but more serious is the so-called infantile type in which there is narrowing of a greater length of the aorta. Patients with this type usually die in early infancy.

The symptoms associated with coarctation of the adult type vary according to the degree of the stenosis and the extent of the collateral arterial pathways. There is usually no difficulty in diagnosis if one is acquainted with the difference between the arterial blood pressure in the arms and in the legs, the palpable pulsations in dilated collateral arteries, the notching of the ribs, and the systolic murmur transmitted down the upper spine. A considerable degree of coarctation usually results in cerebral hemorrhage, thrombosis, heart failure, rupture of the aorta, or bacterial endarteritis. It was because of the incapacity and the high mortality associated with stenosis of marked degree that the following experiments were performed.

The experimental problem which was undertaken was that of severing the aorta at the level of the obliterated ductus arteriosus, closing the two ends, and performing an anastomosis between the divided proximal end of the left subclavian artery and the side of the aorta distal to the point at which it had been divided.

It is known that dogs will not survive total occlusion of the thoracic aorta in one stage if one relies only upon the usual collateral channels. The mortality rate under such conditions is 100 per cent. Hence, if survival should occur in our animals, it would mean that an appreciable quantity of blood was being conducted below the point of occlusion as a result of altering the course of the subclavian artery.

A total of 43 experiments (12) were performed on dogs. Only ten of the animals survived the operation for several months or longer. There were six deaths in the first 24 postoperative hours. Fifteen of the animals lived for periods ranging from five to 25 days. The predominating postoperative complication was paralysis of the posterior part of the body. Limiting our consideration to the ten animals which survived the procedure for long periods, we note that six of these showed no paralysis at any time. Two of the animals had a slight weakness of the posterior extremities which disappeared in a few days. The pressure in the carotid and femoral arteries was determined from time to time. In each instance the arterial pressure in the carotid was higher than that in the femoral but in no case was this difference very great. It appears, therefore, that the flow of blood through the transposed subclavian artery and the dilated collateral vessels was sufficient to prevent death in some instances of total aortic occlusion. Furthermore, it was found that hypotension of the posterior part of the body did not occur in the animals which survived. Further evidence that the transposed subclavian artery was an important pathway for conducting blood to the posterior part of the body was supplied by the one experiment in which the subclavian artery was divided nine months after the original operation. Paralysis of the posterior extremities developed and the animal died 24 hours after operation.

The mortality rate in these experiments and the high incidence of paralysis of the posterior extremities made us hesitate to recommend the use of this procedure in man. In discussing the results Dr. Park and I stated (12), ". . . it would appear that the patient with a chronic partial occlusion of the aorta would tolerate a temporary complete occlusion better than the normal dog in which there has been no stimulus to the formation of collateral arterial pathways. In any case this procedure, or a modification of it, should be considered only in those cases of coarctation in which the outlook is very grave since many patients with coarctation of the aorta have a fairly long life expectancy." After the completion of this work several patients with coarctation of the aorta were studied, but the condition did not seem sufficiently grave to warrant the risk associated with operation. This circumstance was probably fortunate for it appears now that the procedure performed by Crafoord (13) in Sweden (October 19, 1944) and by Gross (14) in Boston (June 28, 1945) is a better one than that devised by Dr. Park and me. In this recent operation the stenotic area is excised and an end to end anastomosis of the two ends of the aorta is performed. Since the lumen of the aorta is considerably larger than that of the subclavian artery, the procedure employed by Crafoord and by Gross seems to be a better one than that which Dr. Park and I described. However, it may be necessary to use our method or a modification of it if the stenotic area is so long, as in the infantile type of coarctation, that the two ends of the aorta cannot be brought together if excision is performed. It is of interest that the method of Crafoord and of Gross when used on normal dogs frequently results in paralysis of the posterior extremities. This observation indicates again that the patient with coarctation is aided in withstanding temporary total occlusion of the aorta by the previous stimulus to the development of collateral pathways.

### PULMONARY STENOSIS AND PULMONARY ATRESIA

It was during a discussion of the experimental studies on coarctation of the aorta with Dr. Helen Taussig that she expressed the opinion that patients with pulmonic stenosis or atresia would be benefited if a means could be devised whereby a greater volume of blood would reach the lungs. Thus we come to a consideration of the third and last group in Abbott's classification, the most prominent feature of which is cyanosis. This third group is a heterogeneous one embracing various types of abnormalities. The most frequently encountered type is the tetralogy of Fallot, which is characterized by pulmonic stenosis or atresia, interventricular septal defect, dextroposition of the aorta, and right ventricular hypertrophy.

I have stated previously that cyanosis is usually present in patients with this and similar abnormalities. It is due to the presence of reduced hemoglobin in the circulating blood and is the visible manifestation of the underlying anoxemia and compensatory polycythemia. Lundsgaard and Van Slyke (15) stated that there are four important factors in the production of cyanosis, namely, (1) the total hemoglobin content, (2) the degree of oxygen unsaturation of the arterial blood coming from the aerated lung areas, (3) the proportion of blood passing from right heart to left through unaerated channels, and (4) the oxygen consumption in the capillaries. It has been found that the cyanosis of most patients, including many of those with congenital cardiovascular defects, can be definitely lessened by the prolonged inhalation of high concentrations of oxygen. The assumption that all of the blood which passed through the lungs was not fully oxygenated led some observers to believe that further increase in the circulation of blood to the lungs would result in no benefit. On the contrary, there are a number of reasons, which will not be repeated here, why patients with pulmonary stenosis or atresia might be benefited if the pulmonary blood flow were increased. Perhaps the most important evidence is supplied by the observation that the condition of children with pulmonary stenosis or atresia and with patent ductus arteriosus becomes worse if the ductus closes, thereby reducing further the flow of blood to the lungs. It is generally recognized by cardiologists that one of the dangers connected with the operative closure of a patent ductus lies in the possibility of an associated pulmonary stenosis, and it is for this reason that the effect of temporary occlusion is tested before the closure is made permanent.

Following the original suggestion of Dr. Taussig some two years ago that a means be found for increasing the flow of blood to the lungs, I undertook studies on experimental animals. It was evident that there were two major problems, namely, (1) the devising of a technique by which the blood flow to the lungs could be increased, and (2) the testing of the method in animals with a high degree of unsaturation of the arterial blood.

Technical methods by which the blood flow to the lungs might be increased will be considered first. There have been reported in the literature only 12 cases of chronic valvular disease in man in which attempts have been made by operative means to lessen the degree of stenosis. One of the patients had pulmonic stenosis, one had aortic stenosis, and the remaining ten had mitral stenosis. The methods which were used consisted of (1) incision of the stenotic area with a small tenotome knife, (2) dilatation of the stenotic area with a finger, and (3) excision of a segment of the valve with a cardiovalvulotome. Seven of the ten operations for mitral stenosis were reported by Cutler and Beck (16). In the first of these patients the stenotic area was incised with a tenotome knife. This patient survived for four and a half years and was believed to have been somewhat improved by the operative procedure. The other six patients reported by Cutler and his associates died shortly after operation. The same was true of the patients with mitral stenosis operated upon by Allen and Graham (17) and by Pribam (18). Souttar (19) reported the survival of a patient in whom he dilated the mitral valve with his finger. The patient of Tuffier (20) in whom dilatation with the finger was used in the treatment of aortic stenosis was said to be improved. Doyen (21) in 1913 reported his experience in the treatment of a patient 20 years of age with congenital pulmonic stenosis. A small tenotome knife was introduced into the right ventricle and an attempt was made to divide what was thought to be a stenotic valve. The patient died several hours later, and examination showed narrowing of the conus rather than stenosis of the valve. Thus it is to be noted that only three of the 12 patients survived the operative procedure, two having had finger dilatation of the mitral or the aortic valve respectively, and one having been treated by incision of a stenotic mitral valve.

It appears to be unlikely that an operation on the stenotic area itself would be successful in the treatment of congenital pulmonic stenosis. The defect is usually in the conus rather than in the valve. Even if one were able to increase the size of the opening by some safe means as yet undiscovered, the chances are that the stenosis would return gradually to the original condition. It was an appreciation of this fact and the previous experience in the anastomosis of the systemic and pulmonic vessels which led to the creation of an artificial ductus arteriosus in the experimental attempt to supply a greater volume of blood to the lungs.

The question arose as to the type of anastomosis which should be performed. A suture anastomosis between the aorta and the main pulmonary artery was excluded as a possibility because it would be necessary to interrupt the circulation for a longer time than is compatible with life. The branches of the aorta which are in such position that they might be anastomosed to one of the pulmonary arteries are the subclavian arteries, the carotid arteries, and the innominate artery. Either the right or the left pulmonary artery might be used. A further question was whether the end of the systemic artery and the side of one of the pulmonary arteries should be anastomosed or whether the end of the systemic artery should be joined to the divided distal end of one of the pulmonary arteries. The former method appeared to be the one of choice since it would allow the blood to flow to both lungs. It is, of course, the type of union which is present in patent ductus arteriosus.

Following these studies on technical means for increasing the flow of blood to the lungs, attention was devoted to attempts to produce in experimental animals the type of general disturbance which is present in the patient with pulmonic stenosis. Means are not available for reproducing in its entirety the tetralogy of Fallot. After unsuccessful attempts to cause cyanosis and anoxemia by several different methods, a high degree of oxygen unsaturation was produced by the removal of lobes of one or both lungs and the creation of pulmonary arteriovenous fistulas by the anastomosis of the proximal ends of the pulmonary artery and vein of the resected lobe or lobes. This operation caused some of the venous blood to return to the left side of the heart without passing through the pulmonary capillaries and resulted in varying degrees of oxygen unsaturation of arterial blood. The creation of an artificial ductus arteriosus under these conditions by the anastomosis of the proximal end of the subclavian or innominate artery to the side of one of the pulmonary arteries usually resulted in an increase in the oxygen saturation of the arterial blood. The presence of the artificial ductus allowed some of the arterial blood which was only partially saturated with oxygen to pass through the lungs instead of continuing through the systemic circulation. While it was realized that the experimentally produced condition was a poor reproduction of that seen in the patient with pulmonic stenosis, the experiments at least supported the hypothesis that improvement would result if an artificial ductus was created.

Even after Dr. Taussig and I decided to attempt the operation on patients, there were three unknown elements which caused hesitation for some time before the initial operation was undertaken. The most troublesome questions were: Would an intensely cyanotic child tolerate a long operative procedure in which general anesthesia and the opening of one of the pleural cavities were necessary? Granting that the answer to the first question was in the affirmative, would the patient tolerate temporary occlusion of either the right or the left pulmonary artery for the time that would be required for making an anastomosis by suture? Would ligation and division of the subclavian artery result in serious impairment of the circulation to the arm? It was apparent that the answers could be supplied only by making the clinical tests. The situation was discussed frankly with the parents of the children who were chosen for operation. A number of distressing complications have been encountered, but experience has shown that the original fears were largely without foundation. By this is meant that thus far all of the children upon whom an operation has been performed have withstood anesthetization, the opening of the pleural cavity, and the temporary occlusion of one of the pulmonary arteries, and have not died during the operative procedure itself. Some subsequent events not so encouraging will be commented upon later. Furthermore, there has been no instance in which the subclavian artery has been used when there has been the slightest anxiety about the competency of the collateral circulation to the arm. Unfortunately the same statement cannot be made in regard to the cerebral circulation in all of the patients in whom the innominate artery has been divided and used for the anastomosis. This subject will be considered later in greater detail.

The selection of patients who are suitable candidates for the operation has been performed by Dr. Taussig and her associates. I should like here to acknowledge the important part which Dr. Taussig has had in all of this work. She is Director of the Cardiac Clinic in The Harriett Lane Home for Children in The Johns Hopkins Hospital and is an outstanding authority on congenital heart disease. Many points, particularly those dealing with diagnosis, which are mentioned only briefly in this lecture, will be considered in detail by Dr. Taussig in her forthcoming book. The two outstanding diagnostic features, both of which should be present if the operation is to be performed, are (1) roentgenographic evidence that the pulmonary artery is small in size, and (2) clinical and roentgenographic evidence of absence of congestion in the lung fields. The important finding in the roentgenogram is the absence of fullness of the normal pulmonary conus. The shadow at the base of the heart to the left of the sternum is concave and not convex; the pulmonary window appears abnormally clear in the left anterior oblique position.

The studies on patients have included determinations of the oxygen content and capacity and percentage saturation of the arterial blood, the red blood cell count, the hemoglobin content, and the hematocrit reading. Patients with inadequate blood flow to the lungs do not necessarily have polycythemia but this was usually observed. The oxygen content of the arterial blood of most of the patients was lower than normal, the oxygen capacity was increased, and hence the arterial saturation was greatly reduced. Although some of the patients were cooperative and quiet at the time of the arterial puncture, most of them were crying and some were struggling. Some of the values for oxygen content would undoubtedly have been higher had the children been under basal conditions. If the arterial saturation was high in a patient with a history of poor tolerance to exertion, the effect of exercise on the saturation was determined in an effort to assess the need for operation. The findings in one such patient were as follows.

This patient was an intelligent and cooperative boy eight years of age. The control figures showed a red blood cell count of 4.7 million, a hematocrit reading of 40.5, an oxygen content of arterial blood of 18.1 volumes per cent, a capacity of 20.5 volumes per cent, and saturation of 88.2 per cent. Because of the history of intolerance of exercise it was decided to repeat the studies after the patient had climbed a few steps. After walking up only six steps, the patient refused to do more and tried to assume the usual squatting position. A sample of arterial blood taken at this time showed an oxygen content of 7.9 volumes per cent and an oxygen saturation of only 36.8 per cent. Operation was decided upon and performed, the innominate artery being anastomosed to the pulmonary artery. Slightly less than three weeks after operation the studies were repeated. The arterial saturation had risen only slightly; that is, from 88.2 per cent preoperatively to 89.7 per cent postoperatively. On the other hand, approximately twice as much exercise as the patient took before operation caused no apparent dyspnea or cyanosis and practically no change in the oxygen saturation, the postoperative figure being 88.2 per cent saturation, whereas the preoperative one was 36.8 per cent. This patient returned for observational purposes on November 2, 1945, three months after the operation. The resting oxygen saturation of arterial blood at this time was 89.2 per cent. The patient then climbed 40 steps without evidence of shortness of breath or cyanosis. A sample of arterial blood taken immediately after the exercise showed an oxygen saturation of 88.6 per cent.

The effect of exercise on the oxygen saturation varies greatly from patient to patient. It is particularly important to study the effect of exercise in patients who are quiet at the time of the control determinations and who have a relatively high resting saturation because the figures may influence not only the decision as to the necessity for operation but also may be the deciding factor in the choice of the artery which is used for the anastomosis.

Another factor which enters into the decision as to operation is the age of the patient. It is more difficult to be certain of the correct diagnosis in infants, and furthermore it is believed that they withstand the operative procedure less well than do children of the group from two to ten years of age. The youngest patient upon whom the operation has been performed was eight months and the oldest 21 years of age. Both of these patients survived the operation and are improved. At the present time it appears that children under 18 months of age should not be operated upon unless it is thought that the chances of survival to an older age are poor. A possible operative procedure that may be perfected for use in small infants will be mentioned later.

If the studies show that an operation is indicated, the patient is given penicillin for one or more days preoperatively. Neither dicumarol nor heparin is given before operation. Cyclopropane with a high concentration of oxygen was used as the anesthetic agent in most cases. In a few of the operations ether with a high concentration of oxygen was used and in others a combination of cyclopropane and ether.[3] After the patient was anesthetized and before the operation was begun, usually a sample of arterial blood was withdrawn. A comparison of the results of the analyses with the control preoperative figures shows that there was an elevation in the oxygen saturation of the arterial blood in all except a few of the patients. This rise ranged from a minimal one to an increase of five times the saturation in the control period. Since many of the children were crying and struggling at the time of the control studies, it is not known whether the elevation in oxygen saturation during anesthesia was due to the fact that the patients were anesthetized and hence quiet or to the inhalation of a high concentration of oxygen. It is probably a combination of the two factors. Most of the patients who were quiet at the time of the control studies did show an elevation of oxygen saturation when given the anesthetic agent and oxygen.

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[3] The anesthetic agent was chosen and administered by Dr. Lamont or Dr. Harmel of the Anesthesia Department. It is a proof of their skill that there have been no deaths during the operation on the 55 patients.

The major details of the operative procedure are contained in the previous report by Blalock and Taussig (22). Adequate exposure is afforded by an anterior incision through the third interspace. There has been some alteration in our conception of the side of the chest on which the operation should be performed. Providing the position of the aorta is normal, it was our earlier idea that the approach should be made on the left if one wishes to use an artery the size of the subclavian artery and that the incision should be on the right if the use of a larger vessel such as the innominate artery is indicated. No doubt you will recall that normally the innominate artery is the first major vessel arising from the arch of the aorta and that it divides into the right common carotid and the right subclavian arteries. The left common carotid artery and the left subclavian artery normally arise separately from the aorta. When the right subclavian artery is used for the anastomosis, experience has shown that the angle of the transposed vessel at its point of origin is less acute than that seen when the left subclavian artery is employed. In other words, the lumen of the vessel appears to be less constricted by the transposed position when the right subclavian is used. For this reason it is advisable usually to make the approach on the right side. This generally allows one to choose either the innominate artery or the right subclavian artery. The preoperative studies will have given a good indication as to the size of the artery that is required. Sometimes, however, the size of the subclavian artery may be smaller or larger than was anticipated and the choice of artery may have to be altered. Furthermore, the innominate artery or the subclavian artery may be shorter than usual and it may be necessary to use the longer vessel regardless of the preoperative choice. The fact remains that the incision on the right side usually allows one to choose either the subclavian or the innominate artery according to the incapacity and the degree of arterial oxygen unsaturation of the patient. It is obvious that the greater the incapacity, the larger the vessel which is needed since the object of the procedure is to shunt blood to the lungs. In infants and small children in whom an operation is urgently needed it is generally advisable to use the innominate artery.

There is another point about the choice of artery which should be mentioned. The occurrence of a right rather than the normal left aortic arch is not rare in patients with congenital cardiovascular malformations which cause cyanosis. Bedford and Parkinson (23) have demonstrated that the determination of the course of the aorta is not difficult if its relationship to the esophagus is delineated under the fluoroscope as a barium mixture is swallowed. The importance of this observation rests on the fact that when the aorta descends on the right, the innominate artery is directed to the left and has to be approached through the left side of the chest. There have been 16 such patients in this series and in all the condition has been diagnosed correctly by Dr. Taussig before operation.

There are many other types of variations in the arteries which arise from the arch of the aorta. For example, both carotids and both subclavians may arise as four separate vessels, no innominate artery being present. In another variation the innominate artery gives rise to both common carotid arteries as well as to the right subclavian artery. Despite the many variations, it has been possible in every case thus far to find a systemic artery which was suitable for anastomosis to a pulmonary artery. There have been two patients, however, in whom a satisfactory anastomosis was not performed because of a variation in the right pulmonary artery. In the first of these the right pulmonary artery divided shortly after its origin and the small branch to the right upper lobe was mistaken for the main artery. This branch was too small for a satisfactory anastomosis. The proximal end of the subclavian artery was anastomosed to the distal end of the branch to the upper lobe, but the size of the subclavian was several times that of the pulmonary vessel. This patient died, and examination of the specimen indicated that the usual end to side anastomosis could not have been performed. Probably the procedure should have consisted of division of the main right pulmonary artery and the anastomosis of its distal end to the end of the innominate artery. In the second patient the right pulmonary artery was not found at the time of operation and it was only after considerable difficulty that it was located at autopsy. The artery was small and was lying inferior and posterior to the superior pulmonary vein.

The pressure in the right or left pulmonary artery was determined at the time of operation in a number of patients by puncturing the artery with a needle which was connected with a water manometer. The pressure in most of the patients was approximately 175 mm. of water. That this procedure should probably be a routine one is suggested by the following experience.

In one of the patients the pressure was 310 mm. of water. In this patient the heart was rotated towards the right, the azygos vein was several times the normal size, and the pulsations of the pulmonary artery were much more vigorous than normal. Despite the unusually high pressure in the pulmonary artery, the end of the innominate artery was anastomosed to the side of the right pulmonary artery. Following operation the cyanosis did not disappear, the liver became enlarged, fluid accumulated in the pleural and peritoneal cavities, and death occurred on the 24th postoperative day. At autopsy a single ventricle was found from which arose both the aorta and the pulmonary artery, and there was little if any pulmonary stenosis. The preoperative oxygen saturation of 59 per cent and the red cell count of 10 million were apparently due to the complete absence of a ventricular septum. It is obvious that the anastomosis should not have been performed, and the high pressure in the pulmonary artery should probably have furnished the clue as to the true nature of the condition.

I shall continue with a description of the operative procedure. After the systemic vessel has been chosen and prepared and the pulmonary artery has been freed from the surrounding structures, usually the end to side anastomosis is performed. The right or left pulmonary artery is occluded proximally with a mechanical device. Distal occlusion of the vessel is produced by making slight traction on braided silk which is placed around the individual branches of the pulmonary artery. This plan as contrasted with the use of a bull dog clamp leaves a greater length of the artery free for the anastomosis. A transverse opening is then made in the pulmonary artery midway between the proximal and distal points of occlusion. By the use of 00000 Deknatel sutures on curved atraumatic needles an anastomosis is performed between the end of the systemic artery and the side of the pulmonary artery. The continuous everting (out, in and over) suture is interrupted at least four times in order not to constrict the lumen too greatly. The procedure is not particularly difficult if the arteries are of normal length and if the respiratory movements are not vigorous. The operation has been described in greater detail in a previous publication (22).

The cyanosis usually increases during the period while one of the pulmonary arteries is being occluded. Following the completion of the anastomosis and the removal of the constricting devices, the color of the patient improves immediately. Usually a distinct thrill can be felt in the pulmonary artery and even in the lung tissue itself. The thrill may be minimal or absent in infants.

Before I leave the subject of technical procedures there are several additional points which might be mentioned. As indicated previously, it is our impression that the union of the end of the systemic artery to the side of one of the pulmonary arteries is the procedure of choice since this type of anastomosis allows the blood to pass to both lungs. There are certain circumstances, however, in which it may be advisable to divide one of the pulmonary arteries and to anastomose the end of this vessel to the end of the systemic artery. This means, of course, that all of the shunted blood passes to one lung and that the blood which passes through the stenosed pulmonary artery goes to the opposite lung. This type of anastomosis can be performed more quickly and easily and with less traction on the mediastinal structures than the one usually employed. It is possible that this method should be used more often, particularly in children. It was used in the following case as well as in three additional ones. The four patients survived the operation and are improved.

A child of slightly less than two years of age had an oxygen content of arterial blood of 3.5 volumes per cent, a capacity of 15.0 volumes per cent, an arterial saturation of 23.3 per cent, and a normal red blood cell count and hematocrit reading. The condition was considered critical and operation was advised. After anesthesia was induced by the use of cyclopropane, the arterial blood pressure fell to 50/30 mm. Hg. An incision was made on the right side of the chest and the azygos vein was doubly ligated and divided. At this time the pulsations of the heart were very weak and barely visible. One half cubic centimeter of coramine was injected into the superior vena cava, and the pulsations of the heart became strong again. The right pulmonary artery was being freed of the surrounding tissues when it was noted again that the action of the heart was poor. Coramine was injected, the pulsations of the heart improved, and it was decided that the operation should be terminated. The child's condition was unchanged after this exploration and a second operation was performed 12 days later. The previous incision was reopened. The innominate artery was shorter than usual, the subclavian artery was larger than usual, and it was decided to use this latter vessel for the anastomosis. When traction was made on the right pulmonary artery, the pulsations of the heart practically ceased. Coramine was injected and the condition improved. In order to avoid unnecessary traction and in order to reduce the operating time, the right pulmonary artery was ligated and divided and its distal end was anastomosed to the end of the subclavian artery. The action of the heart became very weak on two occasions while this was being done, but it responded favorably when coramine was injected. It is doubtful whether the procedure could have been completed successfully if an end to side anastomosis had been performed. The child showed a dramatic improvement. Twelve days following the second operation the arterial oxygen content was 10.1 volumes per cent, the arterial capacity was 16.3 volumes per cent, and the arterial saturation was 62 per cent as compared with the preoperative figure of 23 per cent.

Another point in operative technique which deserves brief mention is the possibility of connecting the lumen of the aorta with that of the main pulmonary artery without the use of sutures. The first portions of the medial walls of the aorta and the pulmonary artery are intimately adherent to each other largely because they are enclosed in a tube of serous pericardium common to the two arteries. In the dog it is possible to connect the two vessels by a stab incision. There are, however, several points in technique which have not been mastered. There is danger of piercing the opposite wall of the aorta, there is no accurate gauge as to the size of the opening, and the fistula, even though moderate in size, may close spontaneously. This problem is still in the experimental stage. It is to be hoped that some such method can be perfected because it may be necessary to use the major blood vessels and to work with considerable speed if newborn infants with pulmonary stenosis or atresia are to be operated upon successfully.

Treatment both during and after the operation will be considered briefly. As a routine procedure a needle is placed in a vein of the ankle before the operation is begun and a very slow infusion of normal saline solution is given. If an abnormally large quantity of blood is lost, plasma is administered. If the patient has pronounced polycythemia and if the loss of blood during the operation is minimal, whole blood equal to approximately one per cent of the body weight is removed at the conclusion of the operation. As soon as the patient is returned to his room he is placed in an oxygen tent. The administration of penicillin which had been begun preoperatively is continued for about two weeks. It is of interest that none of the patients had a postoperative infection. Until recently the administration of dicumarol was begun 24 hours after operation in most cases and was continued for about two weeks. Its use was discontinued when a patient died because of intrapulmonary bleeding. Heparin is not given unless evidence of cerebral thrombosis appears.

The postoperative course of the patients was variable. Those patients who were benefited by the operation showed an early improvement. As soon as the operation was completed, the mucous membranes showed much less cyanosis. It required, however, a longer time for the disappearance of cyanosis of the fingers and toes. The fact that the color of the mucous membranes changes almost immediately following the operation is further evidence of the importance of the volume of the pulmonary blood flow in these patients in the production of cyanosis. In this early postoperative period there has been little alteration in the red blood cell count, the hemoglobin content, and the hematocrit reading even though moderate blood loss has occurred. The one important factor which has been altered is the volume of blood which reaches the lungs for aeration.

I stated previously that there has been no concern about the circulation of the arm, the subclavian artery of which was divided and used for the anastomosis. Sympathetic nerve block was not used in any of these cases. The arm of the operated side was slightly cooler than the opposite one for varying periods of time. Motion and sensation were little if at all affected. The radial pulse has reappeared in some of the patients. Either the right or the left subclavian artery was divided and used for the anastomosis in 26 cases. In 23 additional cases the innominate artery was used and it is apparent that it was necessary to ligate the first portion of the subclavian artery in each of these. Thus, the first portion of either the right or the left subclavian artery was ligated in 49 cases; there was no evidence in any case of dangerous interference with the circulation of the arm. These 49 cases include a duplication in one patient in whom the left subclavian artery was used at the first operation and the innominate artery at the second.

There follows a brief account of the experiences of other surgeons with ligation of the first portion of one or the other of the subclavian arteries. Halsted in 1921 found that the first portion of the left subclavian artery had been ligated in 21 cases. A recent search of the literature provided the records of 13 additional cases in which the first portion of the left subclavian artery was ligated. Of the total of 34 cases, 14 were performed in the treatment of aneurysms, 19 for trauma, and one for a tumor. There were nine deaths in this series. There have been found in the literature references to 57 cases in which the first portion of the right subclavian artery was ligated. The operation was performed because of an aneurysm in 36 cases and because of trauma in 21 cases. There were 24 deaths in this series. It was not necessary to perform an amputation of the arm in any of the patients who survived ligation of the right or left subclavian arteries.

I have stated previously that the innominate artery was used for the anastomosis in 23 of our patients. This fact implies, of course, that one of the common carotid arteries and a subclavian artery were ligated in each of these patients. There were seven deaths in this series. In two of the seven cases the preoperative diagnosis was in error. In two of the remaining five cases death was attributed to cerebral ischemia or thrombosis. Unfortunately an autopsy was not obtained in these two cases. In several additional patients transient weakness of part or all of the opposite side of the body occurred after operation.

A review of the literature from the time of Valentine Mott's operation in 1818 to the present lists 86 cases in which the innominate artery has been ligated. There were 48 postoperative deaths, a mortality rate of 55.8 per cent. There were six cases of complete hemiplegia with four deaths and five cases of partial hemiplegia with two deaths. Eighty of the 86 ligations of the innominate artery have been performed in the treatment of aneurysms. There were 43 postoperative deaths, a mortality of 53.7 per cent. The remaining six ligations were performed in the treatment of trauma. There were five deaths, a mortality rate of 83 per cent. Prior to the initiation of the present work the innominate artery had not been ligated for conditions other than aneurysm and trauma.

In five of our cases in addition to the 23 in which the innominate artery was used for the anastomosis, the common carotid artery was ligated and divided and connected to one of the pulmonary arteries. This artery was used because of an abnormality in the position of the vessels which arose from the aortic arch. There was no sustained evidence of disturbance of the cerebral circulation in these five patients.

Before summarizing the results of the operations performed thus far, I shall relate some of the details of two cases. In the second case of the series the saturation of arterial blood rose from 36 per cent before operation to 83 per cent three weeks later. Five months after operation the saturation was 87 per cent. The red blood cell count dropped from 7.7 to 5 million. The hemoglobin declined from 24 to 17.5 grams, and subsequently to 15.5 grams. In the third case of our series the oxygen saturation rose in nine days from 35.5 to 80 per cent and subsequently to 83.7

per cent. In three and a half months the red blood cell count declined from 10.1 to 5.6 million, the hemoglobin fell from 26 to 13.8 grams and the hematocrit reading changed from 81 to 38. Both of these children were incapacitated before the operation. Now they are able to walk and play and go to school. These two cases are mentioned because the follow-up period has been longer than in others who show equally striking improvement.

I shall attempt now to give the results thus far in The Johns Hopkins Hospital of the creation of an artificial ductus in the treatment of pulmonary stenosis. Up to November 1, 1945, there were 57 operations on 55 patients. All of the operations except one were performed in the nine months' period from February third to November first. An anastomosis between a systemic artery and one of the two pulmonary arteries was not performed in three cases. In two of these there was an anatomical abnormality of the pulmonary artery and in the third there was thought to be an error in diagnosis. The first two patients have died since the operation. The condition of the third patient is unchanged. In the remaining 52 patients, 40 are improved, two have shown little change, and 10 have died.

The deaths will be considered first. The preoperative diagnosis of the tetralogy of Fallot was proved by autopsy to be in error in two of the ten cases. One of these patients had a transposition of the great vessels and the other had a single ventricle without pulmonary stenosis. Theoretically these patients would not have been helped by operation even if they had survived. An autopsy was performed on six of the remaining eight patients and the preoperative diagnosis of the tetralogy of Fallot was confirmed. One could not be certain of the cause of death in most of these patients. Except for the cardiovascular defects the pathological alterations were minimal. None of the patients had an empyema or mediastinitis. As far as could be ascertained, bleeding from the arterial anastomosis did not occur in any of the patients. Two of the deaths were probably due to cerebral ischemia or thrombosis but a postmortem examination of this region was not allowed. One of the patients who had been doing very satisfactorily succumbed suddenly on the fifth postoperative day from pulmonary hemorrhage. The anastomosis was intact, and it is believed that the bleeding was due to the dicumarol that had been given. In one case bilateral pneumothorax developed following the first operation and unilateral tension pneumothorax following the second operation. A congenital abnormality of the lungs was suspected but an examination was not allowed. If the two cases are excluded in which it was shown at autopsy that the diagnosis was incorrect, there were eight deaths in the remaining 50 patients in whom the anastomosis was performed, a mortality of 16 per cent.

It has been stated that two of the patients who survived showed little if any improvement. In one of these, a boy of 15, the intima of the subclavian artery was injured by too vigorous application of the instrument which was used in occluding it. A thrill could not be felt at the end of the operation and a murmur could not be heard later. A second operation on the right side is to be advised in the future. In the second patient a large right pulmonary artery was found at operation and the pulsations were vigorous. The anastomosis was performed even though it was thought at the time that the diagnosis was in error. It has now been seven months since the operation was performed. The parents state that the child is moderately improved. It is our impression that the improvement, if any, as a result of the operation is minimal.

The improvement in the remaining 40 patients is definite. The condition of most of the children has been altered from almost total invalidism to apparent if not real normality. Most of them show no cyanosis, even with moderate effort, and the clubbing of the fingers and toes has disappeared or is diminishing. Some children who had never walked before operation are now able to walk. The average oxygen saturation of arterial blood prior to operation of these patients was 49 per cent and the average saturation when determined two to three weeks after operation was 76 per cent, an average increase of 53 per cent of the control level. It was stated previously that many of the children were not under basal conditions at the time of the arterial punctures and that otherwise probably both the preoperative and postoperative figures would be higher. Although the oxygen saturation of the arterial blood rises greatly, it does not reach the normal value of 95 to 98 per cent saturation. This finding is to be anticipated because in every instance of the tetralogy of Fallot there is an interventricular septal defect and varying degrees of dextroposition of the aorta with overriding of the septum, and hence the aorta always receives some poorly oxygenated blood from the right ventricle. It has been possible, however, in most of these patients to cause an elevation of the arterial oxygen saturation to the point where the stimulus for the development of polycythemia has largely disappeared or has been abolished. The decline in the oxygen capacity has been greater in most patients than has the elevation in the arterial oxygen content. The objective in the performance of the operation is the establishment of a channel by which an adequate quantity of blood can reach the lungs. This pulmonary flow should be adequate to permit growth and the performance of exercise, and to cause the disappearance of polycythemia and cyanosis. It should not be excessive because this will place an undue strain on the heart. There has been only one patient thus far in whom cardiac enlargement since operation has been great enough to cause concern. Dr. Taussig has found that a number of the patients have shown a slight increase in the size of the heart in the early postoperative period, but this enlargement has not been progressive. Streptococcus viridans infection has not developed in any of the patients.

It is important to emphasize the point that the operation is not of value to all patients with persistent cyanosis (22). It is indicated only in malformations in which the primary difficulty is lack of adequate circulation to the lungs. More specifically, the types of abnormalities which should be benefited by the operation are the tetralogy of Fallot, pulmonary atresia with or without dextroposition of the aorta and with or without defective development of the right ventricle, truncus arteriosus with bronchial arteries, and a single ventricle with a rudimentary outlet chamber in which the pulmonary artery is diminutive in size. The operation is not indicated in cases of complete transposition of the great vessels or in the so-called "tetralogy of Fallot of the Eisenmenger type" and probably not in aortic atresia.

The purpose of this lecture has been to discuss certain congenital cardiovascular defects in which treatment by surgical means is proving effective. The incompleteness of knowledge is excusable in view of the only recent application of these methods. It is hoped that the advances recorded thus far will serve as an added stimulus to further studies on cardiorespiratory physiology, including the problem of chronic anoxia, and to the development of additional and improved methods for the treatment of cardiovascular defects.

## BIBLIOGRAPHY

1. White, P. D., Heart Disease, New York, The Macmillan Company, 2nd ed., 1937.
2. Abbott, M. E., Atlas of Congenital Cardiac Disease, American Heart Association, 1936.
3. Munro, J. C., *Ann. Surg.*, 1907, **46**, 335.
4. Graybiel, A., Strieder, J. W., and Boyer, N. H., *Am. Heart J.*, 1938, **15**, 621.
5. Gross, R. E., and Hubbard, J. P., *J.A.M.A.*, 1939, **112**, 729.
6. Keys, A., and Shapiro, M. J., *Am. Heart J.*, 1943, **25**, 158.
7. Touroff, A. S. W., and Vesell, Harry, *J.A.M.A.*, 1940, **115**, 1270.
8. Eppinger, E. C., Burwell, C. S., and Gross, R. E., *J. Clin. Investigation*, 1941, **20**, 127.
9. Keys, A., *Am. J. Physiol.*, 1941, **134**, 268.
10. Gross, R. E., *Surg., Gyn. and Obst.*, 1944, **78**, 36.
11. Levy, S. E., and Blalock, A., *J. Thoracic Surg.*, 1939, **8**, 525.
12. Blalock, A., and Park, E. A., *Ann. Surg.*, 1944, **119**, 445.
13. Crafoord, C., and Nylin, G., *J. Thoracic Surg.*, 1945, **14**, 347.
14. Gross, R. E., and Hufnagel, C. A., *New England J. Med.*, 1945, **233**, 287.
15. Lundsgaard, C., and Van Slyke, D. D., Cyanosis, Medical Monographs, vol. 2, Baltimore, Williams and Wilkins Company, 1923.
16. Cutler, E. C., and Beck, C. S., *Arch. Surg.*, 1929, **18**, 403.
17. Allen, D. S., and Graham, E. A., *J.A.M.A.*, 1922, **79**, 1028.
18. Pribam, B. O., *Arch. f. klin. Chir.*, 1926, **142**, 458.
19. Souttar, H. S., *Brit. M. J.*, 1925, **2**, 603.
20. Tuffier, T., *Tr. Internat. Cong. Med.*, 1913, London, 1914, Sect. 7, Surgery, pt. 2, p. 249; discussion, p. 326, 1914.
21. Doyen, E., 26th Cong. de l'assoc. franc. de chir., *Presse méd.*, 1913, **21**, 860.
22. Blalock, A., and Taussig, H. B., *J.A.M.A.*, 1945, **128**, 189.
23. Bedford, D. E., and Parkinson, J., *Brit. J. Radiol.*, 1936, **9**, 776.

# THE CHEMICAL EVOLUTION OF VISION[1]

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## I. ARGUMENT

THE theory of evolution is founded primarily upon morphological data. In its approach to such data it is guided always by the fundamental distinction between analogous organs, which, whatever their basic composition, serve similar functions; and homologous organs, which, whatever their functions, share deep-seated correspondences in structure, origin, and position in the organism. Only homologies are accepted as a proper indication of biological relationship.

As the biologist penetrates to smaller and smaller elements of the organism, he reaches finally, and as we now know with no essential discontinuity, the molecular level. Here physiology fuses with physical chemistry; and morphology becomes one with chemical structure. On this level again one can readily distinguish analogies and homologies (cf. 52). The physical chemistry of biological systems, taken by itself, leads only to analogies. Their chemical structure and origins provide the stuff of homologies. As such they open the possibility of pursuing biological relationship in the realm of molecular dimensions. In this sense one can speak of chemical evolution.

Throughout the past half-century the realization has grown that the processes which govern stimulation by light in all types of organisms—animal and plant—have many characteristics in common; and that underlying these is a common plan of physicochemical organization. In all cases one has to deal with a pseudo-reversible system, in which a photosensitive pigment is acted upon by light, and is reconstituted by ordinary "dark"—that is, thermal—reactions. The light reaction results in excitation; the reverse processes permit continued response in the light and recovery in darkness.

[1] Lecture delivered December 20, 1945.

This concept of the photoreceptor process was discussed with relation to animal vision by the physical chemist Luther and his colleagues (43, 44), who were led to it by their investigations of pseudo-reversible photochemical systems *in vitro.* It was soon afterward applied by Blaauw (3) in a closely reasoned analysis of parallelisms between human vision and phototropic bending in a unicellular mold and in the oat shoot. Hecht (27) retraced the history of these ideas before the Harvey Society a few years ago, and it is he who has principally developed them and impressed them upon our generation. The beautiful experiments of Hartline (24), also lately reviewed for the Harvey Society, show that these viewpoints are as applicable to the responses of single elements of invertebrate and vertebrate retinas as to the whole organs.

This is clearly an argument on the level of molecular analogy. It tells us that photoreceptors wherever found and however composed satisfy certain basic physicochemical requirements, just as an effective wing, whether in insects or birds, must conform with certain aerodynamical principles.

The anatomical examination of photoreceptors also leads only to analogies. No morphological correspondences can be traced among the photoreceptors of lower and higher plants, the integumentary photoreceptors of lower invertebrates, and the various types of invertebrate and vertebrate eyes.

It has lately become evident, however, that throughout this entire range of organisms photoreceptor systems are bound together in a profound chemical homology. From the systems which initiate bending in fungi and higher plants to the eyes of mammals they depend regularly, perhaps universally, upon one distinctive and compact group of substances, the carotenoids.

They are related also in a further sense for which there exists no morphological counterpart. Though one speaks of morphological inheritance, there is of course no actual transmission of anatomical structures from one organism to another. There is very commonly, however, a passing on of molecules which organisms require yet cannot themselves synthesize, and which they must therefore obtain from other organisms in the diet.

Photoreceptor systems display this type of connection also. At some point in the evolution of animal photoreception the capacity to synthesize carotenoids is lost. So far as we know, all animals with eyes must obtain the carotenoids which they employ in vision from other organisms, ultimately from plants. In this process the very molecules which take part in plant phototropism pass into animals to be incorporated into the structures of their visual systems. It is this relationship which brings vitamins into the chemistry of vision. The term is arbitrarily reserved, however, not for the unchanged plant carotenoids, which animals use in vision only as accessory substances, but for degraded derivatives of the plant carotenoids, the vitamins A.

The most instructive homologies indicate not only correspondences, but distinctions. Fortunately this is the case with the chemical homology which we are discussing. Within the over-all pattern of carotenoid utilization, plants, which make their own carotenoids, are divided off from animals which have lost this capacity. With the appearance of animal organisms one begins to find also specific modifications of plant carotenoids, the so-called zoocarotenoids, whose full status in photoreception one cannot yet evaluate. In animals with eyes such modifications of ingested plant carotenoids have produced the vitamins A. Finally, within the vertebrates, a cleavage appears between the stocks which use vitamin $A_1$ and those which use vitamin $A_2$ in vision.

It is the purpose of this essay to trace the pattern of these relationships. They came to be appreciated only as a by-product of the chemistry of visual systems. They have never been pursued for their own ends as systematically as they deserve. This fact will, I hope, explain in part obvious gaps and inadequacies in portions of the argument.

## II. PLANT PHOTOTROPISM

The bending of plants toward light is stimulated primarily by the blue region of the spectrum. It is very different in this regard from photosynthesis. Thus Sachs (54), in 1864, showed that sunlight, after passing through saturated dichromate solution, is highly active in photosynthesis, but has no phototropic effect whatever on a variety of etiolated seedlings. Sunlight which has passed through a blue cupric ammonium filter produces only very feeble photosynthesis, yet is highly active in stimulating bending.

It is a direct consequence of the Law of Conservation of Energy that light, to produce a chemical or physical effect, must first be absorbed; in acting upon a material system it must be transformed to some other form of energy, and hence must cease to exist as light. The blue-sensitivity of phototropic plants must therefore be associated with their possession of blue-absorbing—that is, yellow—receptor substances.

This inference appears not to have been drawn explicitly for a long period. In 1930, however, Bachmann and Bergann (1) suggested that the phototropic sensitivity of oat seedlings in the spectrum resembles the absorption of light by a "chromolipoid," i.e., a carotenoid. This view has since been developed by Voerkel (60), Castle (12), Bünning (8, 9, 10) and others.[2]

The carotenoids form a group of fat-soluble, highly unsaturated, yellow to red pigments, very widely distributed in animals and plants. They consist primarily of long hydrocarbon chains in which single and double bonds alternate to form what is known as a conjugated system. It is to this arrangement that the carotenoids owe their color. Carotenoids which contain only carbon and hydrogen are called carotenes, from their most familiar member, the principal pigment of the carrot. Carotenoid alcohols, which contain various numbers of hydroxyl groups, are known as xanthophylls. The predominant carotenoids found in green plants and animal tissues are β-carotene, $C_{40}H_{56}$, and lutein or leaf xanthophyll, $C_{40}H_{54}(OH)_2$. Their structural formulae follow on page 121.

The lavishness with which carotenoids are synthesized in plants, their wide distribution, and their close association with the chlorophylls in all types of photosynthetic cells have prompted the suspicion that they must play some critical role in plant metabolism. With almost negligible exceptions, no such place can yet be assigned to them (72). It has at various times been proposed that carotenoids participate in oxidoreduction systems, that they protect other structures in the tissues from oxidation or destruction by light, or that they contribute significantly to the synthesis of chlorophyll. None of these functions has been satisfactorily established. Nor is the light absorbed by the carotenoids effective in photosynthesis in the higher green plants, green algae or photosynthetic bacteria which have been examined. There exists as yet only an indirect indication that carotenoids may participate with very low efficiency in the photosynthesis of the blue-green alga, *Chroococcus* (17); and one instance, involving the diatom *Nitzschia,* in which light absorbed by carotenoids appears to be used in photosynthesis with an efficiency approaching that of chlorophyll (15).

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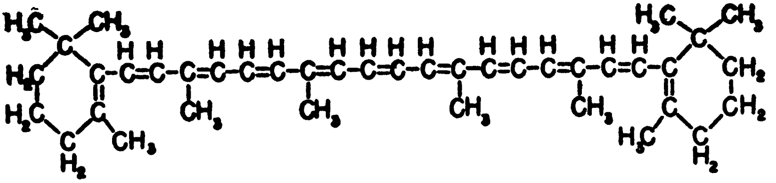
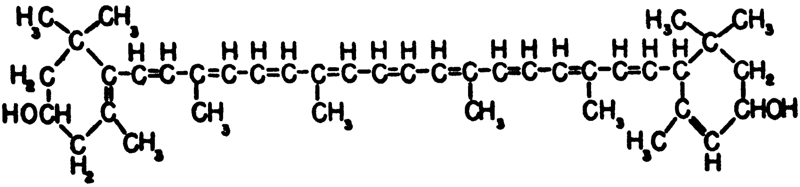
[2] A more extended discussion of the position of carotenoids in plants will be found in reference (72).



*β-carotene*, $C_{40}H_{56}$



*lutein*, $C_{40}H_{54}(OH)_2$

The only function in plants in which carotenoids seem to play a general and primary part is that of photoreception in systems concerned with orientation to light—phototropic bending, directed movements of free-swimming forms, light-growth responses, and chloroplast migrations. The belief that carotenoids act in this way rests upon the following considerations.

Carotenoids have been found in all phototropic structures in which they have been sought. They are present in large amounts in all green plants, including etiolated plants which contain no chlorophylls. The etiolated coleoptile of the oat seedling (*Avena*), a classic object in the study of phototropism, contains both carotenes and xanthophylls, principally the latter (75, 9). No other pigments appear in this tissue. Bünning (9) has reported, furthermore, that in the oat coleoptile carotenoids are wholly restricted to the photosensitive zone. The unicellular sporebearers of the molds *Phycomyces* and *Pilobolus*, the phototropic reactions of which have also been studied intensively, contain carotenes alone (55, 12, 9); and here also Bünning finds them concentrated in the light-sensitive zone.

Among all the pigments which occur in phototropic structures, only the carotenoids possess absorption properties consistent with the observed spectral sensitivities. The latter indicate a maximum absorption in the blue, falling off in the violet, and declining very steeply at longer wavelengths to negligible values in the green, yellow and red. This type of absorption is not found in chlorophyll *a* or *b*, bacteriochlorophyll, protochlorophyll, phycoerythrin or phycocyanin (72).

A few instances in which the phototropic sensitivity has been measured in some detail throughout the spectrum reveal a remarkable degree of correspondence with the absorption spectra of the associated carotenoids. This relationship is illustrated in figure 1. The lower portion of the figure shows the spectral sensitivities for phototropic bending in the mold *Pilobolus* (8) and in the oat shoot (29). In both instances two maxima appear. In the upper part of the figure are shown the absorption spectra of the total carotenoids of the oat coleoptile and of *Phycomyces*;[3] the spectrum of an extract of *Pilobolus* was not available, but

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[3] Absorption spectra are presented in terms of the percentage of light absorbed, or of the extinction or optical density, log $I_0/I$, in which $I_0$ is the incident and I the transmitted intensity of radiation.

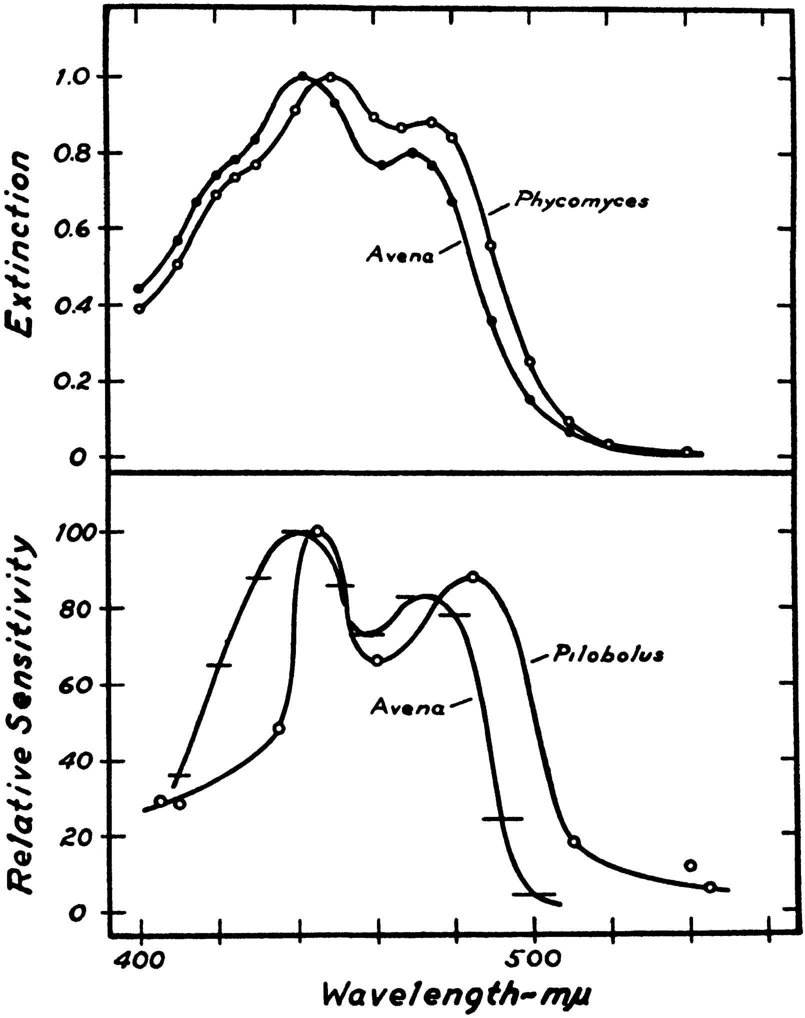

FIG. 1. Spectral sensitivity for phototropic bending of plants, and absorption spectra of the associated carotenoids. *Above:* Absorption spectra of the total carotenoids of the etiolated oat coleoptile (*Avena*), and of spore-bearer cells of the mold *Phycomyces* (72). *Below:* Spectral sensitivity of the oat shoot (29), and of spore-bearers of *Pilobolus* (8). It is known that the carotenoids of *Pilobolus* and *Phycomyces* are virtually identical (9).

Bünning has shown that both molds possess apparently identical carotenoids.

The marked similarities in form and position of the carotenoid absorptions and the phototropic sensitivities strongly support the view that the latter depend upon the former. Particularly impressive are the parallel displacements of both pairs of functions. The difference in the absorption spectra reflects the fact that the principal pigment of *Phycomyces* or *Pilobolus* is β-carotene, while that of the oat coleoptile is leaf xanthophyll. The parallel shift of the phototropic spectra is convincing evidence that they depend upon the carotenoid absorptions.

Phototropic bending is not the only type of light reaction in plants which appears to be mediated by carotenoids. Bottelier (6) has measured the spectral sensitivity for inhibition of protoplasmic streaming in the epidermal cells of the oat coleoptile; it agrees in general form and position with the phototropic spectrum, and hence with carotenoid absorption. Voerkel (60) has measured in broad spectral regions the energies required to induce a constant state of chloroplast orientation in the moss *Funaria;* again the sensitivity follows a course consistent with the absorption of light by carotenoids. The photic orientation of free-swimming green organisms also is of this general nature, but for reasons about to be explained these are discussed separately.[4]

In a relatively small number but wide variety of plant structures, therefore, the sensitivity of oriented reactions to light is greatest in the blue region of the spectrum, less in the violet, and negligible in the yellow to red. Carotenoids are the only pigments in these structures whose absorption of light is consistent with this distribution of sensitivity. In certain instances the spectral sensitivity mimics in some detail the absorption spectra of the extracted carotenoids (*Avena, Pilobolus*). In some structures also (*Avena, Pilobolus, Phycomyces*) the carotenoids are concentrated in or restricted to the photosensitive zones. In all these cases it is reasonable to conclude that the primary process of photoreception is mediated through carotenoid pigments. This is as yet the only general statement that can be made of the function of carotenoids in plants.

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[4] Engelmann (18) has described a type of response to light radically different in nature from those discussed above. This is found in certain motile aerobic cells which either depend for their motility upon oxygen or are chemotactic to it. In situations in which the oxygen is supplied primarily by photosynthesis, whether of the cells themselves or of associated plants, the spectral sensitivity for motion or orientation corresponds with that for photosynthesis. Engelmann carefully distinguished such responses, which are really chemotropisms, from the genuine ''Lichtempfindungen,'' of the type discussed above. There is nothing to prevent a given organism from exhibiting both types of reaction—chemotropism to oxygen with a spectral sensitivity based upon chlorophyll, and genuine phototropism, based upon the carotenoids.

## III. GREEN FLAGELLATES

These unicellular and colonial organisms are included among algae by botanists, who are principally struck by their possession of chloroplasts, and among protozoa by zoologists who are impressed by their frequent lack of other plant characteristics. Because of this equivocal status and their special arrangements for responding to light I shall discuss these organisms separately.

Ordinarily they contain a structure which seems to be concerned specifically with photoreception, the stigma or eye-spot. This view was questioned by Engelmann (18), who, from the behavior of *Euglena viridis* at the edges of sharp shadows, concluded that the light-sensitive zone is localized in the colorless region just anterior to the stigma. It is doubtful that shadows to be obtained in the field of a microscope are sufficiently sharp-edged to permit such fine discrimination. Later workers who have re-examined this matter have tended to agree with the original investigators that the stigma is in fact the photoreceptor organ (46, 42).

The most direct evidence that this is so comes from measurements of the spectral sensitivity for phototropic orientation of these organisms. Mast (45) has provided a careful and detailed series of such measurements. White light projected at a right angle to narrow spectral bands from a monochromator was adjusted in intensity so as to induce a fixed angle of orientation in the organisms. The results were afterward corrected for the energy distribution of the source. A number of *Euglena* species which were so examined (*viridis, tripteris, gracilis, granulata* and *minima*) all proved to be maximally sensitive in the blue, at 473–483 mμ. The same was true of *Phacus triqueter, Trachelomonas euchlora* and *Gonium.* The sensitivity maximum of *Chlamydomonas* lay in the blue-green, at 504 mμ; while those of *Pan-*

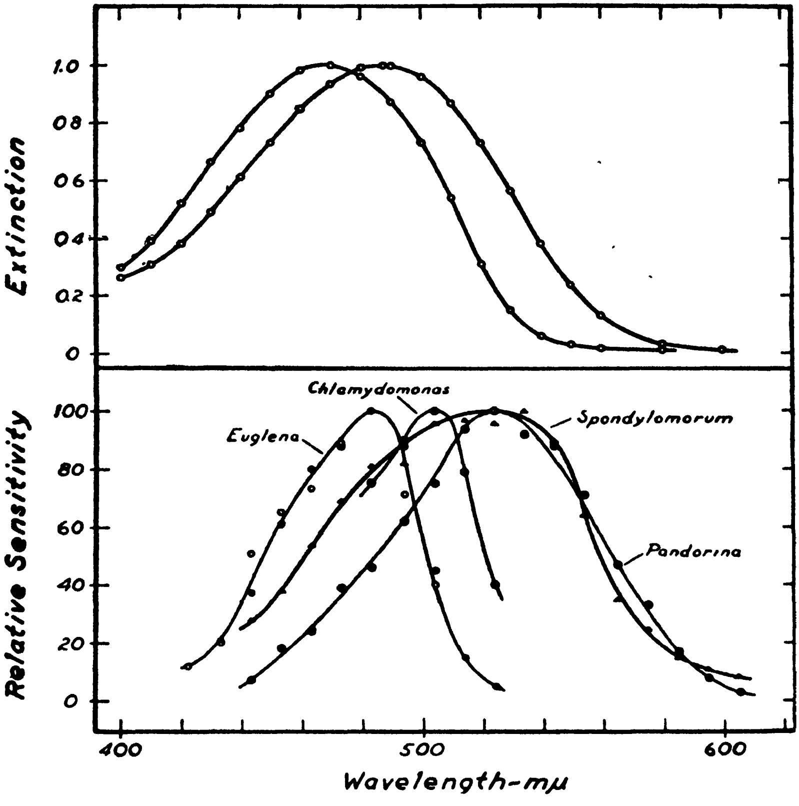
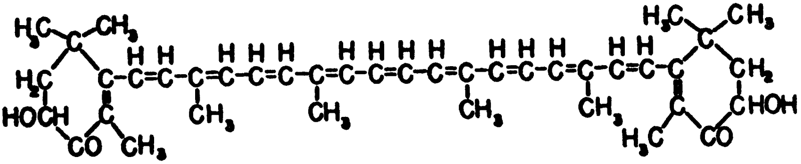


FIG. 2. Spectral sensitivity for photo-orientation of green flagellates and absorption spectra of astaxanthin. *Above:* Spectra of astaxanthin dissolved in hexane (left) and in castor oil (right) (72). *Below:* Spectral sensitivities of *Euglena viridis, Chlamydomonas globulosa, Spondylomorum quaternarium* and *Pandorina morum,* from Mast (45).

*dorina*, *Eudorina* and *Spondylomorum* were in the green, at about 534 mμ. In all cases the sensitivity falls more or less symmetrically to both sides of the maximum. A representative group of these data is shown in figure 2. They are in good general agreement with other work on these and similar organisms (45, 38, 42).

All these measurements agree in two general characteristics: the photo-orientation spectra all possess single maxima, and are displaced toward considerably longer wavelengths than are associated with phototropic bending.[5] A pigment possessing the absorption characteristics indicated by these spectral sensitivities should be orange, red or purplish-red in color. It is an important justification of the belief that the stigma is the photoreceptor organ that it does in fact have colors in this range.



*astaxanthin*, $C_{40}H_{52}O_4$

The principal carotenoid of green flagellates has enjoyed a long history under a variety of names: the ''haematochrome'' of Cohn, ''carotinin'' of Zopf, and ''euglenarhodon'' of Tischer (58, 59). It is apparently concentrated in the stigma and may also be dispersed throughout the other chromoplasts. It has recently been identified as astaxanthin (35).

This substance possesses the structure of a dihydroxy-diketo-β-carotene. Spectra of astaxanthin published by Kuhn and Sörenson (34), which display three maxima, are in error. Repeated measurements in our laboratory have shown the spectra of astaxanthin and its esters to consist of a single broad band which lies at considerably longer wavelengths than those of the common carotenes and xanthophylls. It conforms therefore in general character with the spectral sensitivities observed in green flagellates (figure 2).

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[5] The only exception to this statement of which I know involves Blaauw's measurements on *Phycomyces nitens*, not since confirmed, showing a maximum phototropic sensitivity at about 495 mμ (3). Recent measurements on *Phycomyces blakesleeanus* all agree in showing a sensitivity maximum in the neighborhood of 440 mμ (11, 7, 10).

How widespread the possession of astaxanthin is among these organisms is not yet known. It has been identified in *Hematococcus* and *Euglena,* in company with smaller amounts of the common plant carotenoids (58, 59, 35). Possibly more extended examination of the green flagellates will reveal the presence of other special pigments.

Meanwhile it may be noted that the range of phototropic sensitivities exhibited by these organisms is not beyond the capacities of astaxanthin itself. In simple solution the maximum absorption of this pigment and its esters varies between about 470 mμ in hexane to about 492 mμ in pyridine. In ordinary cell oils the maximum lies at about the same wavelength as does the phototropic sensitivity of the *Euglenas* (figure 2). In still denser media, in suspension, or in the solid state the spectrum is further displaced toward the red. Astaxanthin also readily forms complexes with protein, which range in color from its native red to green, as in the ovoverdin of lobster eggs (34, 57), and blue, as in lobster shells (35). A comparable array of conditions would permit astaxanthin to meet the most extreme requirements of phototropic sensitivity in the green flagellates.

Quite apart from its possible role in photoreception the very appearance of astaxanthin in these organisms provides a curious chemical commentary upon their biological status. For astaxanthin is an *animal* carotenoid. It has not otherwise been found in any but animal tissues, where, so far as known, it is produced by modification of ingested plant carotenoids. The equivocal position of the green flagellates among animals and plants therefore extends to the molecular level. They possess at once the exclusively plant pigment, chlorophyll, and the exclusively animal carotenoid, astaxanthin.

## IV. EYELESS INVERTEBRATES

To the extent that green flagellates are admitted within this category we have already considered the most substantial body of information which it contains. Orientations to light abound among the lower invertebrates, but few accurate measurements have been made of the associated spectral sensitivities, and nothing is known of the associated pigmentation.

Loeb was concerned over a period of years to establish the essential identity of plant and animal phototropism. In plants, phototropic bending is accomplished by differential growth. Stimulated by Sachs's analysis of this process, Loeb (40) examined the mechanism of bending in a number of attached invertebrates. He found that the polyps of certain hydroids, *Sertularia* and *Eudendrium,* bend toward light as do plants, not by contraction, but by differential growth. Years later, Loeb and Wasteneys (41) repeated Blaauw's *Avena* experiments on newly formed polyps of *Eudendrium ramosum.* Lines of these organisms were exposed for various periods to the carbon arc spectrum, and were then replaced in darkness and the proportions which responded by bending observed. In the spectrum, uncorrected for energy distribution, the effect was sharply maximal in the blue at about 474 mμ, decreased in the violet, and was negligible in the orange and red. This result is almost identical with Blaauw's uncorrected observations on the oat shoot and implies a corresponding dependence of photoreception upon carotenoids. Unfortunately we lack as yet the crucial demonstration that *Eudendrium* contains these pigments.

In general the photic responses of lower invertebrates are pitched at longer wavelengths than those associated with phototropic bending, either of plants or of *Eudendrium.* They are in fact included roughly within the range of action spectra observed within the green flagellates. Mast (45) found the phototactic sensitivies of the earthworm and of larvae of the marine worm *Arenicola* to be maximal at about 483 mμ, resembling in form and position those of the *Euglenas.* Hecht (25, 26) measured the relative energy in broad regions of the spectrum required to elicit the photic responses of the clams *Mya* and *Pholas* within a constant reaction time. The sensitivity of *Mya* is maximal in the blue-green, at about 490 mμ, falling steeply to both sides; that of *Pholas* in the green, at about 555 mμ, with possibly an added maximum in the ultra-violet.

None of this information contributes materially to the problem under discussion. Before it can do so some systematic study must be made of the pigments of photosensitive structures in this great range of organisms. Such an exploration offers the opportunity to bridge what is now an enormous gap in the knowledge of photoreceptor systems.

It promises in addition an insight into the genesis of the vitamins A. As the thread of the present argument is lost among lower invertebrates, these substances have not yet appeared. When it is resumed in the eyes of arthropods and molluscs, animals have come to depend on plants for their carotenoids, and have developed the capacity to degrade plant carotenoids to yield the vitamins A. The latter substances hereafter dominate the photoreceptor process. It should be important to locate the origins of this critical development.

## V. VERTEBRATE RODS: THE RHODOPSIN SYSTEM

The first image-forming eyes are found in arthropods and molluscs. Within these phyla they differ radically in construction. The principal eyes of arthropods are of the compound or mosaic type, composed of a large number of independent units, isolated from one another by screening pigments, and each possessing its own optical system. The only molluscan eye discussed below—that of the squid—contains a large lens and a retina composed of a single layer of cells, their photosensitive segments turned toward the light. Vertebrates also have a lens eye; but with a multi-layered retina in which the receptor cells face away from the light, and are of two kinds: rods, concerned with vision in dim light, and cones, the organs of vision in bright light and color vision.

In spite of such wide anatomical differences, the photoreceptor processes in all these eyes operate with a very limited group of chemical substances and a very restricted pattern of reactions. For this reason it is advantageous to consider at once the visual systems which have been most thoroughly explored, those of the vertebrate rods.

Franz Boll discovered in 1876 the first photosensitive pigment of the retina in the rods of frogs (5). It was the rose-colored substance named *rhodopsin* shortly afterward by Willy Kühne. In one extraordinary year Kühne learned almost everything that was known until very recently of the chemistry of this substance (36).

In the living animal rhodopsin bleaches in the light and is resynthesized in the dark, and so fulfills the elementary physicochemical requirements of a photoreceptor pigment. In aqueous solution the absorption spectrum of rhodopsin consists of a single broad band, maximal at about 500 mμ (figure 5). It agrees very well in shape and position with the spectral sensitivity of human rod vision (66).

Rhodopsin is a conjugated protein, which owes its color and most distinctive properties to a carotenoid prosthetic group (61). It bleaches in the light in a complex succession of photic and ordinary thermal reactions to orange or yellow products (66). At some point in this process carotenoid is split from protein, and the original prosthetic group of rhodopsin is transformed to the stable yellow carotenoid, retinene$_1$.

This substance has never been found in other tissues than the retina. In solution in chloroform its spectrum consists of a broad band, maximal at about 387 mμ (figure 5). Carotenoids in general form violet to blue-green products when treated with antimony chloride. Retinene, in this reaction yields a deep blue color, due to a specific absorption band at 664 mμ (figure 3, b).[6]

In the intact retina the mixture of retinene$_1$ and colorless protein which results from bleaching reverts in part to rhodopsin; most of the retinene$_1$, however, is converted irreversibly to vita-

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[6] It has recently been suggested that retinene$_1$ is the aldehyde of vitamin A$_1$, but the evidence for this is very indecisive (28, 50).

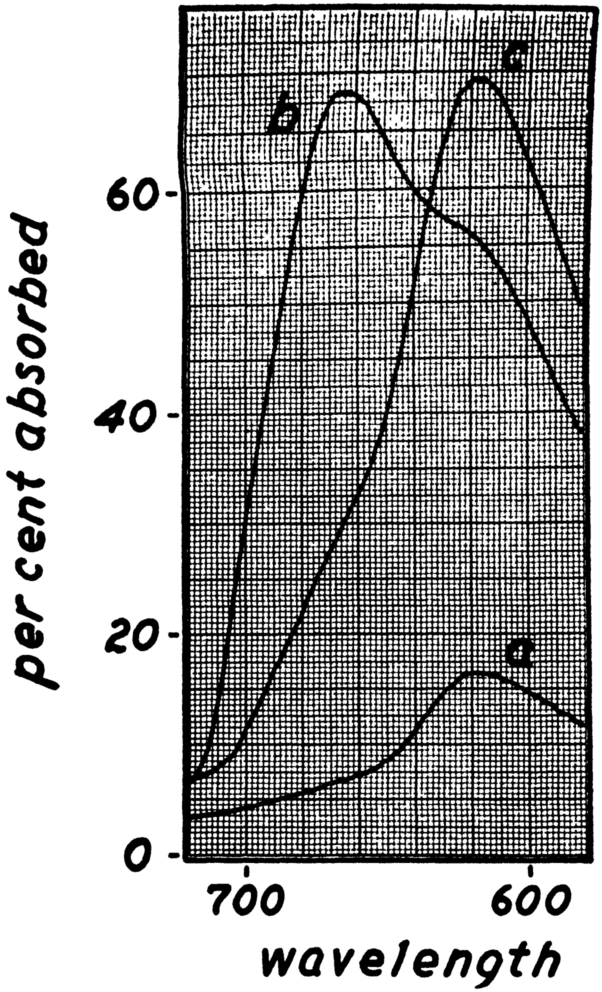
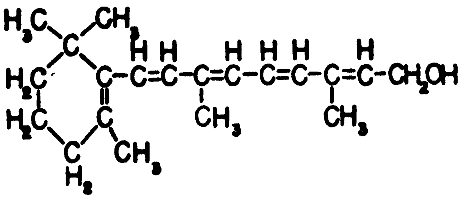


FIG. 3. The rhodopsin system. Spectra of the antimony chloride reaction with extracts of retinas of the smooth dogfish. (a) Dark adapted retinas contain a small amount of the 618 mμ-chromogen, vitamin $A_1$. (b) Immediately following irradiation these same tissues yield a large quantity of the 664 mμ-chromogen retinene$_1$, liberated in the bleaching of rhodopsin. (c) In irradiated retinas allowed to remain for about an hour before extraction the retinene$_1$ has been converted to vitamin $A_1$ (68).

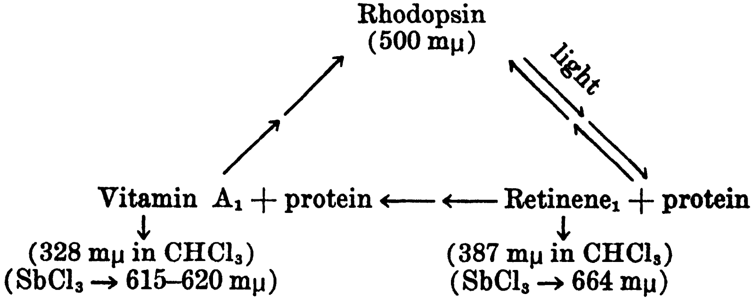
min $A_1$. This substance has the structure of half a β-carotene molecule to which the elements of water have been added:



vitamin$A_1$, $C_{20}H_{29}OH$

It possesses an absorption maximum in chloroform at about 328 mμ (figure 5), and yields with antimony chloride a blue color due to an absorption band at 615–620 mμ (crude tissue extracts; figure 3, a, c).

In the isolated retina, continued exposure to light leads to the irreversible formation of a mixture of vitamin $A_1$ and protein. But in the intact animal this mixture in turn is resynthesized to rhodopsin. The result is a closed cycle of the skeletal form (61, 62):



The operation of this retinal cycle can be demonstrated in a simple procedure which has become standard in our laboratory. Data from such an experiment are shown in figure 3.[7] Right and left retinas of dark adapted animals are prepared separately in dim red light. One set of retinas is shaken thoroughly in the dark with petroleum ether, an operation that does not disturb rhodopsin. The colorless extract, tested with antimony chloride, yields a low absorption band at about 618 mμ, showing that the dark adapted retina contains a small amount of free vitamin $A_1$ (figure 3, a). This same tissue is now exposed to bright light, which instantly bleaches the rhodopsin to an orange color, and is immediately re-extracted exactly as before with petroleum ether. The extract is now yellow, and yields when mixed with antimony chloride a high absorption band at 664 mμ, showing that the bleaching of rhodopsin has liberated a large quantity of $\text{retinene}_1$ (figure 3, b). Meanwhile the symmetrical set of retinas has also been exposed to light, but is allowed to remain at room temperature for about an hour before extraction. During this period the retinas fade to colorlessness. Extracted with petroleum ether exactly as before, they yield a colorless solution, which in the antimony chloride test displays a high absorption band at 618 mμ (figure 3, c). In the process of fading, $\text{retinene}_1$ has been completely removed, and has been replaced by an equivalent quantity of newly formed vitamin $A_1$.

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[7] The spectra shown in figures 3, 4, 10, 11 and 12 are recordings drawn automatically with Hardy's photoelectric spectrophotometer at the Massachusetts Institute of Technology. They have merely been mounted for publication. Figures 6, 7 and 8 show tracings from such recordings.

In figure 3 this experiment has been performed with retinas from the smooth dogfish. Identical results have been obtained from several genera of bony fishes, from frogs, chickens, rats, rabbits, and cattle. A recent examination of light adapted human retinas revealed the presence of vitamin $A_1$ here also (74). The constitution of the rhodopsin cycle principally accounts for the now familiar association of night-blindness with human vitamin A-deficiency.

## VI. INVERTEBRATE EYES

Until recently vitamin A had not been identified in a single invertebrate tissue, and there was some ground for the suspicion that invertebrates lack this factor. Gross hauls of marine zooplankton, composed almost entirely of small invertebrates, were found to contain little or no vitamin A, and so little of its known carotenoid precursors as to make it problematical how fishes which feed on zooplankton can accumulate their large stores of this vitamin (14, 20). It had been reported also that the cockroach and clothes moth do not require vitamin A in their diets (47, 13).

It is now known, however, that in a number of invertebrates high concentrations of vitamin $A_1$ and retinene$_1$ appear in the eye (69, 72). In the vertebrate body most of the vitamin A is concentrated in the liver; that such small amounts are found in invertebrates is probably associated primarily with the lack of such a storage tissue. But beyond this, there is as yet no evidence that in invertebrates vitamins A have other functions than in vision.

Among molluscs only the retina of the squid, *Loligo pealii*, has been examined (69, 71a). It contains about 1–2 μgm. of vitamin $A_1$ per eye, and about three times this quantity of retinene$_1$ (measured as relative extinction in the antimony chloride test). Most of the retinene is bound in a photostable complex which probably represents a retinal reserve. No trace of these or other carotenoids was found in other squid tissues or in extracts of whole bodies less the retinas. The *visual* significance of the carotenoids appears with peculiar force in these animals.

The concentration of retinal vitamin $A_1$ remains constant in all conditions of light and darkness. No evidence was found that it participates at all in the visual process.

The exposure of dark adapted retinas to light, however, always liberates a considerable amount of retinene$_1$. The squid appears to possess a reduced retinal cycle of the form: photopigment $\xrightleftharpoons{\text{light}}$ retinene$_1$ + —————. Attempts to extract the photopigment into aqueous solution have not yet succeeded. Judging from measurements of the spectral sensitivity of the squid retina, however, the photopigment possesses absorption properties resembling those of rhodopsin (Hartline, personal communication; cf. also (4)). The deep purple color of squid retinas, sometimes erroneously ascribed to "visual purple" is due to a light-stable, alkali-soluble, probably melanoid pigment (19). Other than screening the retinal cells this probably has no function in vision.

In the squid, therefore, retinene$_1$ appears to pre-empt completely the vitamin A function. It alone appears to be active in vision; and it is also stored in high concentration in the retina. It is in fact the vitamin A of the squid.

No arthropod systems have yet been examined as completely as this. For the most part experiments have been confined to the fractionation and identification of the carotenoids of the eye. In a number of marine crustacea—the green and fiddler crabs and the lobster—the eyes contain very high concentrations of vitamin $A_1$ in addition to astaxanthin and other carotenoids found throughout the integument. No trace of $retinene_1$ has as yet been found in these eyes. In a freshwater crustacean, the crayfish *Cambarus virilis,* the eyes contain both vitamin $A_1$ and $retinene_1$ in addition to astaxanthin (69, 72).

The eyes of all the invertebrates examined therefore contain vitamin $A_1$, and certain of them also $retinene_1$, in relatively high concentration. These substances reappear in the vertebrate retina as components of the rhodopsin system. In addition, astaxanthin and smaller amounts of other carotenoids occur in the eyes of crustacea; but unlike the vitamins A they are widely distributed also throughout the integument of these animals, and apart from screening the retinal cells they appear to play no active role in vision.

## VII. THE PORPHYROPSIN SYSTEM

In the course of his classic investigations, Kühne made a practice of looking at the dark adapted retinas of every kind of animal he could lay hands on. It was an impressive collection, ranging from the lamprey to man. He noticed that while the retinas of mammals, owls, frogs and so on were rose-colored, due to their content of rhodopsin, those of fishes appeared distinctly *purple* in color (36, 37). Years later Köttgen and Abelsdorff (32) showed that solutions of retinal photopigment from eight species of fish had different spectral properties from those of all other vertebrates examined.

In a first attempt to pursue this matter further several years ago, I examined the retinas of a number of marine fishes and was surprised to find that they possess typical rhodopsin systems (63). It developed that the German workers had invariably used *freshwater* fishes in their experiments. On turning to these I had no difficulty in confirming their observations. The photopigment of the freshwater fish retina is purple in color. In aqueous solution its spectrum consists of a single broad band, maximal at about 522 mμ (Fig. 5). I have suggested that it be called *porphyropsin.*

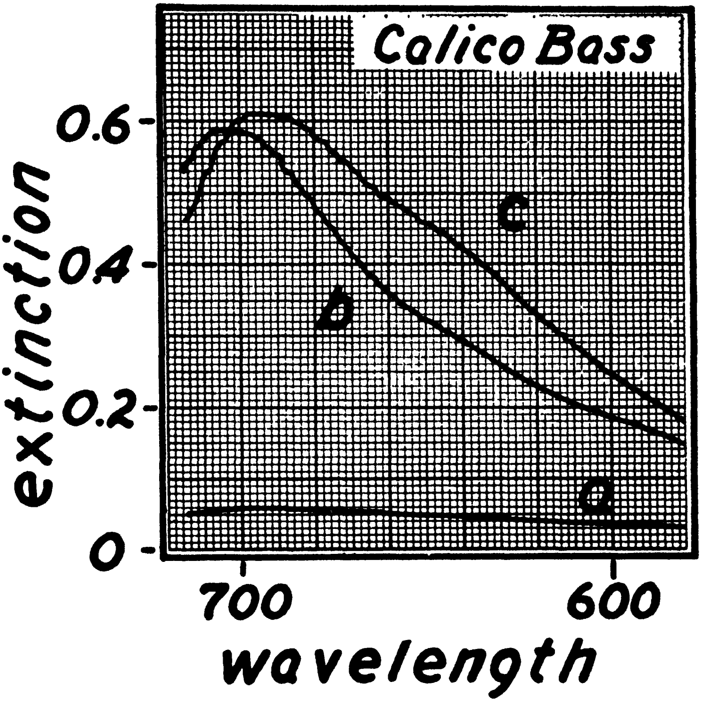

FIG. 4. The porphyropsin system. Spectra of the antimony chloride reaction with extracts of retinas of the freshwater calico bass. (a) Dark adapted retinas yield a trace of the 696 mμ-chromogen, vitamin $A_2$. (b) Immediately following irradiation the same tissues yield a large quantity of the 705 mμ-chromogen retinene$_2$. (c) In irradiated retinas allowed to remain for about one hour before extraction the retinene$_2$ has been converted to vitamin $A_2$ (68).

Like rhodopsin, porphyropsin is a conjugated protein. Its behavior in the retina or in solution is in every detail parallel with that of rhodopsin. It is involved in a retinal cycle of precisely the same form. Yet all components of the porphyropsin cycle have absorption spectra displaced toward the red from their analogues in the rhodopsin system.

The relation between the two cycles was revealed by repeating the experiment shown in figure 3 with retinas of a freshwater fish (figure 4). The extract of dark adapted retinas yields when mixed with antimony chloride, instead of a low vitamin $A_1$ band at 618 mμ, a similar low band at 696 mμ (curve a). Re-extraction of these retinas following irradiation yields, not the $\text{retinene}_1$ band at 664 mμ, but a similar high band at about 705 mμ (curve b). Retinas bleached and allowed to fade for an hour before extraction yield, instead of newly formed vitamin $A_1$, a large amount of the 696 mμ-chromogen. It is clear that in the porphyropsin system a 705 mμ-chromogen replaces $\text{retinene}_1$, and a 696 mμ-chromogen replaces vitamin $A_1$ (64, 68).

On the basis of these retinal observations it was suggested that the 696 mμ-chromogen be called vitamin $A_2$ (39, 16). One may similarly refer to the 705 mμ-chromogen as $\text{retinene}_2$. The porphyropsin cycle then takes the form (64, 68).

$$\begin{tikzcd}
 & \begin{matrix}\text{Porphyropsin}\\ (522\ \text{m}\mu)\end{matrix} \arrow[dr, shift left, "\text{light}"] & \\
\text{Vitamin } A_2 + \text{protein} \arrow[ur] \arrow[d] & & \text{Retinene}_2 + \text{protein} \arrow[ul, shift left] \arrow[ll] \arrow[d] \\
\begin{matrix}(355\ \text{m}\mu \text{ in } CHCl_3)\\ (SbCl_3 \rightarrow 696\ \text{m}\mu)\end{matrix} & & \begin{matrix}(405\ \text{m}\mu \text{ in } CHCl_3)\\ (SbCl_3 \rightarrow 705\ \text{m}\mu)\end{matrix}
\end{tikzcd}$$

The direct spectra of known components of the porphyropsin system are shown in figure 5. Vitamin $A_2$ in chloroform solution possesses a maximum at about 355 mμ, $\text{retinene}_2$ at about 405 mμ. A roughly constant separation of 20–30 mμ therefore divides the spectra of all these substances from their analogues in the rhodopsin cycle. The significance of this difference is reasonably clear. In homologous series of carotenoids or synthetic polyenes the addition of one conjugated double bond is known to shift the spectrum 20–30 mμ toward the red (33, 31). All the known relations between the rhodopsin and porphyropsin systems are satisfactorily explained if the latter possesses such an added double bond in its carotenoid residue.[8]

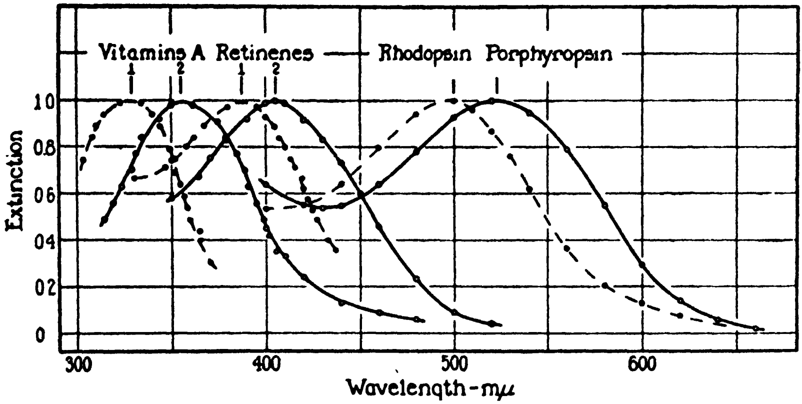

FIG. 5. The rhodopsin and porphyropsin systems. Direct spectra of crude preparations from retinas of the marine scup (broken lines) and the freshwater calico bass (solid lines). Rhodopsin and porphyropsin are dissolved in 1 per cent aqueous digitonin solution, the retinenes and vitamins A in chloroform. All maxima have been brought to the same height to facilitate comparison (68).

This clarification of chemical relationships between the rhodopsin and porphyropsin systems left their biological status still very obscure. A division between marine and freshwater fishes on this basis receives no support from taxonomy; for freshwater fishes commonly have closer relatives in the sea than in their home streams and ponds. Is this then a direct response to the environment? And how precisely is this division maintained?

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[8] The structure of vitamin $A_2$ is still very uncertain. The original view that it is the next higher homologue of vitamin $A_1$ (Gillam, Heilbron, *et al.* (20a)) has been abandoned. Karrer and Bretscher (30) have lately proposed for it on open-chain structure, which would give it much the same relation to lycopene, the pigment of the tomato, that vitamin $A_1$ bears to β-carotene.

To answer the latter question I undertook a survey of the visual systems of marine and freshwater fishes. The results can be expressed most simply in terms of the antimony chloride tests with completely bleached retinas, which contain the vitamins A alone. The presence of the appropriate vitamin A has proved to be a reliable indication of the operation of the entire rhodopsin or porphyropsin system; and in almost all instances the complete cycles were examined (63, 68).

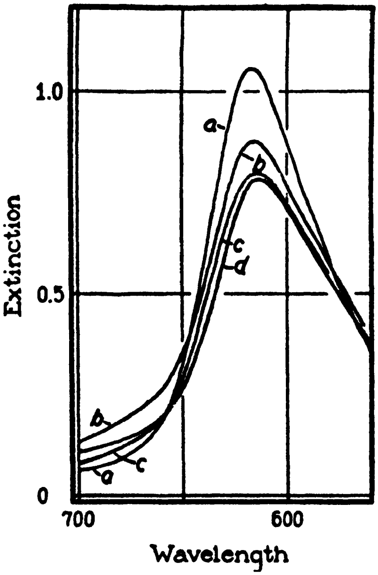

FIG. 6. Retinas of marine fishes. Spectra of the antimony chloride reaction with extracts of bleached retinas from (a) the sea bass, (b) scup, (c) sand flounder, and (d) sea robin. These tissues contain vitamin $A_1$ alone (67).

The retinas of a wide variety of marine cartilaginous and bony fishes were found to contain vitamin $A_1$ alone (figure 6). The forms examined include by now the smooth and spiny dogfishes, the torpedo, swordfish, sea robin, sea bass, scup, sand flounder, herring, haddock and whiting. The only exceptions were found in the family of wrasse-fishes (*Labridae*)—the cunner and tautog—the retinas of which contain a great predominance of vitamin $A_2$.

Conversely, the retinas of a variety of freshwater bony fishes contain only vitamin $A_2$ (figure 7).[9] The catfish, yellow perch, pickerel, carp, calico bass, goldfish, and blue-gill were examined. So far no exception has been found among these forms.

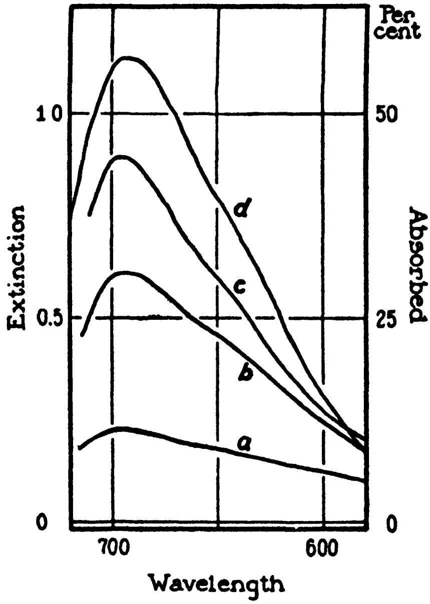

FIG. 7. Retinas of freshwater fishes. Spectra of the antimony chloride reaction with extracts of bleached retinas from (a) the pickerel, (b) calico bass, (c) white perch, and (d) carp. Ordinates for curves (a) to (c) are extinction, for (d) percentage absorption. These tissues contain only vitamin $A_2$ (67).

For the very reason that this division is so sharp, it lends a critical interest to those fishes which are neither freshwater nor marine, but migrate freely as adults between the two environments. Furthermore, only these fishes, since they partake of both environments, can decide the crucial problem whether the vitamin A pattern is determined by the environment or genetically.

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[9] The white perch was included in this figure before its special status as an anadromous fish was appreciated (see below).

## VIII. THE EURYHALINE FISHES

To understand what follows it will be necessary to consider first the biological position of these forms. The fishes which were discussed above are known as stenohaline—i.e., sharply restricted to a narrow range of salt concentration, whether freshwater or marine.

The euryhaline fishes, on the other hand, tolerate a wide range of salinities. What has been emphasized in the past has been their habit of migrating between fresh water and the sea. They are divided on this basis into two groups: *anadromous* (literally, upstream) fishes, like the salmons, which make spawning migrations from the sea to fresh water; and *catadromous* (i.e., downstream) fishes, like the "freshwater" eel, which migrate in the reverse direction.

The emphasis on migration in these forms, however, misses the fundamental distinction between them. For none of these fishes has to migrate; migration is for them merely a potentiality which the various stocks realize in varying degree. Thus among the "anadromous" salmonids, the Atlantic and chinook salmons regularly go to sea, the rainbow trout does so only rarely, while the common brook trout is intermediate in this regard, becoming in migration a sea trout. Similarly many habitual migrators, like the Atlantic salmon or the alewife, are found land-locked in freshwater lakes, where they have spent many generations in the one environment.

The basic distinction in the life histories of euryhaline fishes is that they are rigidly restricted in the environment in which they spawn and develop embryonically. For all of them the spawning environment can be the permanent habitat. The salmon is really a freshwater fish able as an adult to migrate to sea, the eel a marine fish which can come as an adult into fresh water. It would do no violence to the situation to regard both types as transitional between the permanently marine and freshwater fishes. I shall therefore give the terms applied to euryhaline fishes this more basic if less literal meaning: anadromous fishes are those which spawn in fresh water, catadromous fishes are those which spawn in the sea.

Data from the retinas of three genera of salmonids and from the freshwater eel are shown in figure 8. All these fishes possess mixtures of vitamins $A_1$ and $A_2$, of the rhodopsin and porphyropsin systems. The anadromous salmonids possess a predominance of the porphyropsin system, the catadromous eel predominantly rhodopsin. Not all euryhaline fishes yield this kind of result.

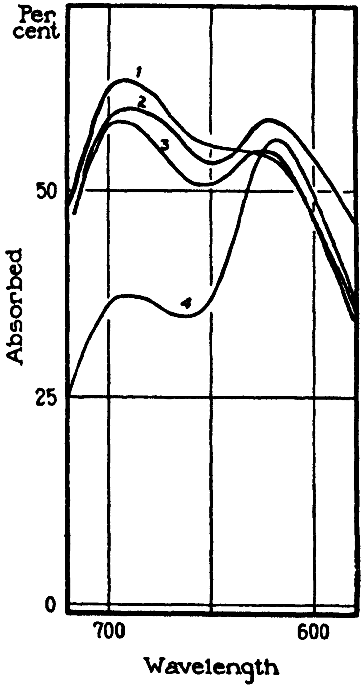


FIG. 8. Retinas of euryhaline fishes. Antimony chloride reaction with extracts of bleached retinas from (1) the chinook salmon, (2) rainbow trout, (3) brook trout, and (4) the "freshwater" eel, *Anguilla*. All these tissues contain mixtures of vitamins $A_1$ and $A_2$, the anadromous salmonids predominantly the latter, the catadromous eel predominantly the former (67).

Some anadromous forms—the alewife and white perch—possess exclusively porphyropsin (cf. figure 7, c, figure 9). All the euryhaline fishes so far examined, however, follow this simple rule: they possess predominantly or exclusively the vitamin A and visual system ordinarily associated with their spawning environment (67, 70).

A remarkable parallelism exists, therefore, between a graded series of salinity relations and the distribution of visual systems. This is illustrated in terms of retinal photopigments in figure 9.

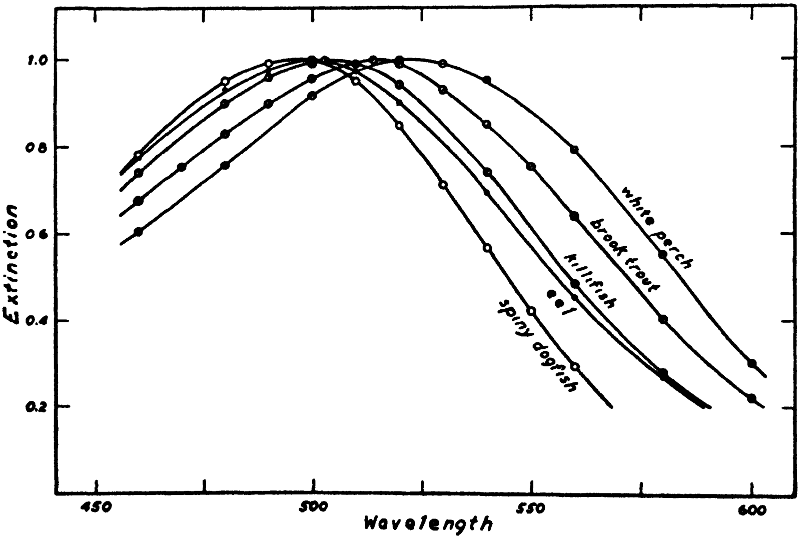


FIG. 9. Spectra of photosensitive pigments from the retinas of fishes, illustrating the transition from an exclusively rhodopsin to an exclusively porphyropsin system. The permanently marine dogfish possesses only rhodopsin, the catadromous eel and killifish predominantly rhodopsin, the anadromous brook trout predominantly porphyropsin, and the anadromous white perch only porphyropsin (70).

Beginning with a typical rhodopsin spectrum from the permanently marine spiny dogfish, the spectrum shifts regularly toward longer wavelengths in the eel, a marine fish which migrates into fresh water; the killfish, which usually compromises by passing its whole life in brackish water; the brook trout, a freshwater fish which can enter the sea; and the white perch, an anadromous fish which has completed the transition to porphyropsin.

These patterns are genetic, and to a first approximation independent of the environment. The salmonids which yielded the data of figure 8 were obtained from a fish hatchery and had never been exposed to salt water, yet they all possess large amounts of the marine rhodopsin system. The eel retains a great predominance of vitamin $A_1$ after years in fresh water, the alewife exclusively vitamin $A_2$ after years in the sea. Eels and permanently freshwater fishes taken from the same pond possess almost diametrically opposed vitamin A configurations in their retinas. Finally, the Labrid fishes, exceptional in uniting permanent marine existence with a predominance of $A_2$ in the retina, furnish final proof that racial type and not environment determines this distribution.

These distinctions divide a number of species which are very closely related taxonomically. The herring and alewife are both *Clupeids;* the herring is permanently marine, the alewife spawns in fresh water and ordinarily spends all but a few months of its existence in the sea. Yet alewives just in from the ocean possess only vitamin $A_2$ in their eye tissues, while the herring eye contains vitamin $A_1$ alone; the liver oils display parallel differences. Similarly the marine black sea bass and the anadromous white perch are both *Serranidae;* the eye tissues of the former contain only vitamin $A_1$ and rhodopsin, those of the latter only vitamin $A_2$ and porphyropsin.

Since this correlation is genetic, its meaning must be sought in the phylogeny rather than the physiology of the fishes. There exists considerable anatomical and paleontological evidence for the belief that fishes originated in fresh water (56, 53). Such forms as produced the modern lungfishes probably have remained continuously in this environment. Most other stocks appear to have ramified widely into the oceans during the late Paleozoic and early Mesozoic, some of them to return later to fresh water. How often such interchanges between the two environments have since occurred, there is no way of knowing. It is in any case very likely that modern freshwater teleosts have all passed through some period of marine existence.

It is with such evolutionary migrations between fresh water and the sea that the vitamin A pattern seems to be correlated. The permanently freshwater fishes use vitamin $A_2$ in vision, the permanently marine forms—with the known exception of the Labrids—vitamin $A_1$. The euryhaline fishes occupy an intermediate position both in adult habitat and in vitamin A pattern; and both characteristics are correlated predominantly with the spawning environment.

## IX. ORIGINS OF VERTEBRATE VISION—THE LAMPREY AND LUNGFISH

The foregoing discussion seems to leave the freshwater fishes in a peculiarly isolated position. They are, so to speak, bracketed between the invertebrates with eyes and all the other groups of vertebrates, all of which use components of the rhodopsin system in vision. Their own choice of the porphyropsin system has the appearance of a curious evolutionary aberration.

Yet the freshwater fishes have a major place in vertebrate evolution. As already noted, it is believed that the vertebrate stock originated in fresh water; and freshwater fishes almost surely also furnished the ancestors of the amphibia. Here are two directions in which one might look for some further extension of the porphyropsin system.

Among the lowest living chordates, below the vertebrates themselves, one finds such animals as *Balanoglossus,* the tunicates, and *Amphioxus,* none of which, for want of well organized eyes, can contribute to our problem. But among the Cyclostomes, the most primitive of living vertebrates, are found the lampreys, animals with very considerable eyes. They offer the closest available approach to the origins of vertebrate vision.

All the lampreys—like the presumed ancestral vertebrates—spawn in fresh water. The form which I examined, however, the sea lamprey (*Petromyzon marinus*) is particularly interesting from the present viewpoint, for it is anadromous. After a larval period of several years in fresh water, it metamorphoses and migrates seaward, returning to fresh water some two years later to spawn. I have examined the retinas of sexually mature animals taken from a tidal estuary in the early stages of the spawning run. If their long sojourn in the ocean had had any effect, it might have inclined them to the rhodopsin system.

The sea lamprey, however, possesses a great preponderance of the porphyropsin system, like an anadromous fish. The force of this comparison was strengthened by a curious circumstance. Our lampreys were accompanied in their spawning run by alewives, anadromous fish coming upstream from the sea for the same function. Though these animals are widely separated phylogenetically, their vitamin A patterns are almost indistinguishable (71).

The retinal use of vitamin $A_2$ therefore appears to extend as far backward toward vertebrate origins as it is possible to penetrate. It probably is as ancient as the vertebrate stock itself. There is as yet no evidence that it extends below the vertebrates. It will be recalled that the only freshwater invertebrate examined, the crayfish, possesses retinene$_1$ and vitamin $A_1$, as do marine invertebrates. It is possible that vitamin $A_2$ and the porphyropsin system are vertebrate innovations.

If so, they join at the point of origin of the vertebrates another, now famous, example of chemical evolution, discovered by Meyerhof and his colleagues (49). This is the transition from the use of arginine-phosphoric acid to creatine-phosphoric acid as a source of energy for muscular work. Almost all invertebrate muscles possess the arginine compound alone; while all vertebrates investigated and the protochordate *Amphioxus* have only the creatine compound. It has since been reported that only echinoderms among the invertebrates, and the primitive chordate *Balanoglossus,* contain mixtures of arginine and creatine phosphoric acids (51, 2). These are precisely the types which are most commonly believed on morphological grounds to mark the path of chordate origin.

I have been able also to examine the retinas of the African lungfish, *Protopterus*. This animal is believed to have descended from a line of continuous freshwater ancestry, widely separated from that of the modern freshwater teleosts, and close to that of the amphibia. The available data indicate that this animal also uses vitamin $A_2$ in vision (71a).

The association of the porphyropsin system with existence in fresh water and particularly with freshwater spawning therefore appears to be very deeply embedded in vertebrate history. It is a short step from finding this system in the lungfish to looking for it in the amphibia; but this consideration raises a number of new problems.

## X. AMPHIBIA: THE NEWT AND FROG

If the habit of spawning in fresh water is basically associated in vertebrates with the retinal utilization of vitamin $A_2$, one should expect to find the porphyropsin system within the amphibia. Most of these animals spawn in fresh water just as do freshwater and anadromous fishes. Yet rhodopsin was discovered originally in the rods of frogs, and all the fundamental chemistry of the rhodopsin system has since been worked out in this animal.

I have come to regard the amphibia as strikingly parallel in biological position with the euryhaline fishes. Both groups are characterized by migrations between fresh water and another environment, which in fishes is the sea, in amphibia the land. Both groups display fixed spawning environments, for the most part fresh water ("anadromous"), though exceptional "catadromous" animals lay their eggs in the sea (freshwater eels) or on land (certain frogs and salamanders). The metamorphoses which are so prominent an accompaniment of migration in some of the amphibia are by no means lacking in the euryhaline fishes. All the salmonids show great changes in body color on going from fresh water into the sea, and salmons in the spawning migration undergo profound changes in anatomy, skeletal and otherwise. The eel goes through such a striking metamorphosis before entering fresh water that for a long period the larval eel or leptocephalus was thought to belong to a distinct genus.

The parallels between amphibia and euryhaline fishes are therefore rather impressive. It is difficult to avoid the inference that such an "anadromous" amphibian as the frog should have primarily the porphyropsin system. That it entirely lacks this system poses something of a dilemma.

The tail-less amphibia—frogs and toads—are among the most highly specialized and aberrant members of the class. It seemed worthwhile, therefore, to examine one of the relatively generalized urodeles. We turned to the common newt, *Titurus viridescens,* which has an anadromous life history. Sexually mature adults in the "water phase" were examined. The retina was found to contain vitamin $A_2$ alone, like many anadromous fishes (71a). The liver also appears to contain only vitamin $A_2$. This marked the first appearance of $A_2$ above the fishes, and in continuing association with the habit of freshwater spawning. By the same token it aggravated the position of the similarly anadromous frog.

The beginning of the War interrupted this line of experiment, and we should have come no further with it but for a fortunate accident. On an excursion in New Hampshire in 1942 we found a pond along the shore of which large numbers of bullfrogs (*Rana catesbiana*) were metamorphosing. In the shallow water were the large tadpoles in all stages of metamorphosis, while along the bank were numbers of the newly emerged frogs. We collected groups of these animals and brought them back to the laboratory.

A first experiment was done with tadpoles approaching metamorphosis, and with just-emerged frogs. The tadpoles all had small hind legs, about ½ to 1 cm. long; no front legs had yet appeared. The antimony chloride tests with extracts of the completely bleached retinas of both groups of animals are shown in figure 10. The tadpole retinas contain a large amount of vitamin $A_2$, with only a trace of $A_1$. The frog retinas present just the reverse pattern.

The dark adapted retinas of these tadpoles also appeared distinctly purple in color, while those of the small frogs were bright rose. Extracts of the photosensitive pigments of the re-

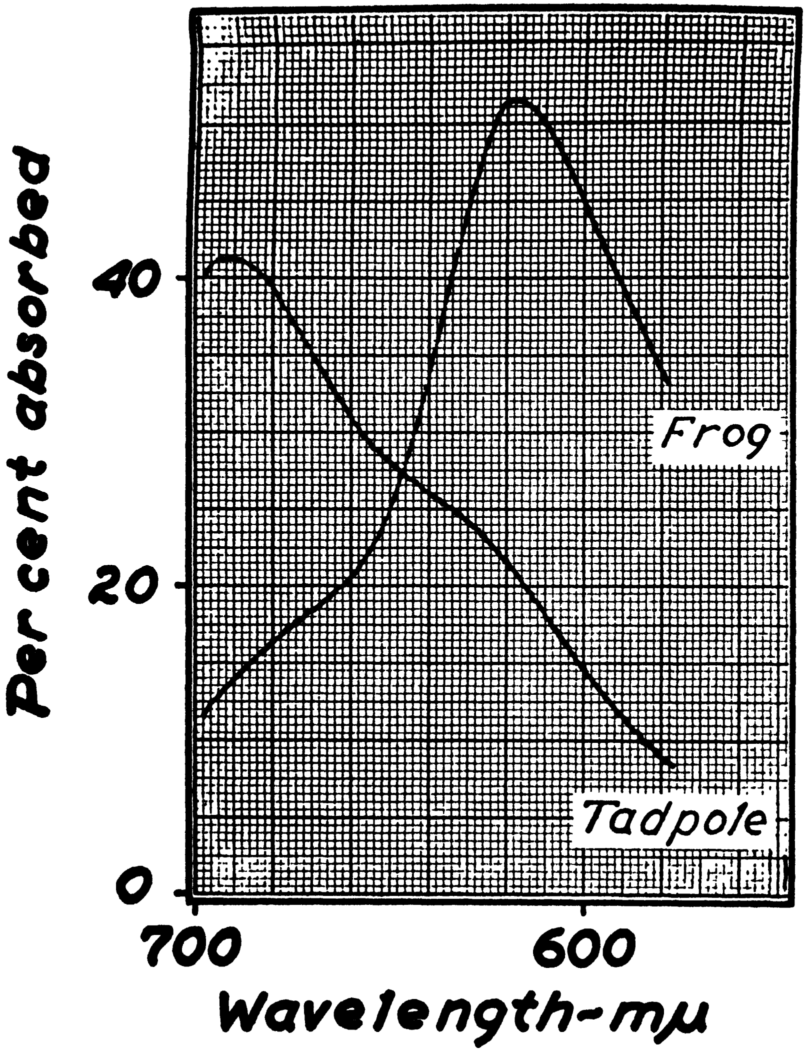


FIG. 10. Transfer from the porphyropsin to the rhodopsin system during metamorphosis of the bullfrog. Antimony chloride tests with extracts of bleached retinas from tadpoles approaching metamorphosis, and from newly emerged frogs. The tadpole retina contains a high concentration of vitamin $A_2$ with only a trace of $A_1$, the frog retina just the reverse pattern (74).

tinas of these animals were prepared. The frog pigment had the familiar absorption maximum at 500 mμ characteristic of rhodopsin. The tadpole pigment was purple in color, and its absorption spectrum was maximal at about 516 mμ, as though porphyropsin mixed with a little rhodopsin were present.

I had also seven animals which were all in about the same advanced stage of metamorphosis. All had both front and hind legs well developed, but all still had long tails, and two still had the tadpole suckers, while in the others the mouth had begun to widen. Antimony chloride tests with extracts of bleached retinas and pigmented layers (pigment epithelium and choroid) of these animals are shown in figure 11. The retinas contain almost equal amounts of vitamins $A_1$ and $A_2$; the pigmented layers are further along in the transition to $A_1$.

The bullfrog therefore possesses as a tadpole the porphyropsin system, and precisely at metamorphosis changes over completely to the rhodopsin system which characterizes the adult. In intermediate stages of metamorphosis it possesses mixtures of both systems, such as we have encountered previously only among the euryhaline fishes.

It is difficult to view this phenomenon otherwise than as a recapitulation. Indeed the morphological changes in metamorphosis can be—and commonly are—so regarded. They appear to retrace, though surely in abridged and corrupt form, the changes which accompanied the evolutionary passage of vertebrates from fresh water to the land. Associated with this migration—as with the passage seaward—vertebrates returned to the use of vitamin $A_1$ in vision. This is the evolutionary transformation which the metamorphosing bullfrog retraces.[10]

[10] Porphyropsin is not the only molecule to show such changes in the bullfrog. McCutcheon (48) has measured in this animal the oxygen dissociation curve of hemoglobin during and after metamorphosis. In the tadpole the curve is hyperbolic and the oxygen-affinity is high. During metamorphosis the curve takes on an inflected S-shape and the oxygen-affinity falls greatly. These changes continue for several months after metamorphosis has been completed. There is no clear argument for regarding them as a chemical recapitulation. Yet it is curious that changes in the same direction are found in the hemoglobins of the developing chick (23) and the mammalian fetus (22).

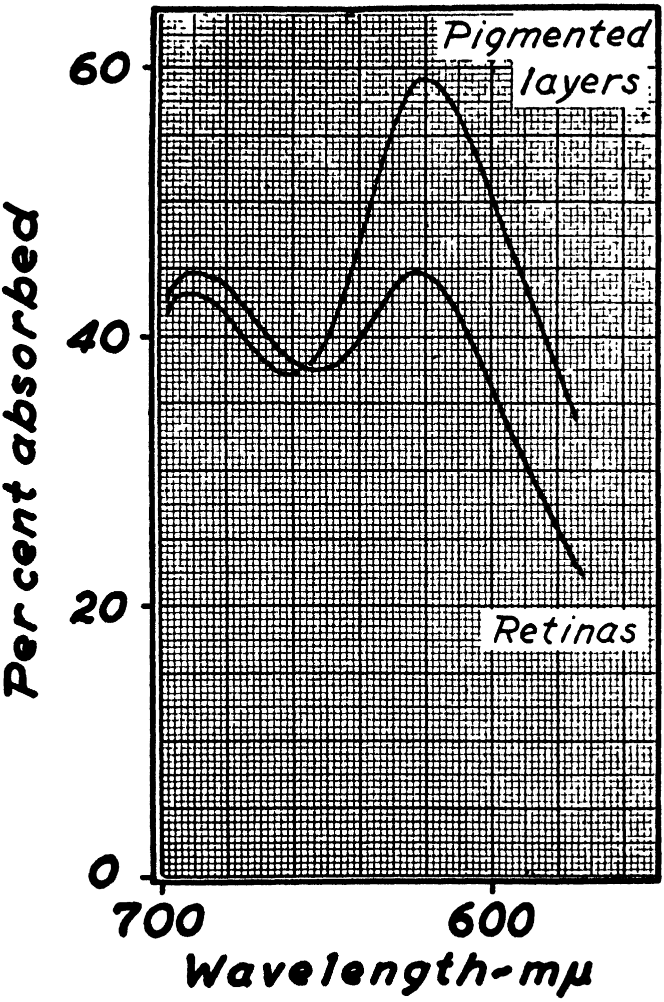


FIG. 11. Vitamins A in the eye tissues of the partly metamorphosed bullfrog. Antimony chloride tests with extracts of bleached retinas and of the combined pigment epithelium and choroid layer of animals which had fore- and hind-legs, long tails, and in most of which the mouth had begun to widen. The retinas contain almost equal amounts of vitamins $A_1$ and $A_2$, the pigmented layers a predominance of $A_1$ (74).

This also completes what is known of the evolution of rod vision. We have as yet no information on the rod vision of reptiles; but all the rods of birds and mammals so far investigated possess only the rhodopsin system. Yet with this, the history of vertebrate vision is not quite ended, for nothing has yet been said of the cones.

## XI. CONE VISION

Though rod photopigments have been known for about 70 years, until recently there was no direct evidence of analogous substances in the cones. It was clear that such pigments must exist, and since none were visible, that they must occur in very low concentrations. For this reason I chose for a first attempt to extract cone photopigments the retina of the chicken, which contains many more cones than rods.

In extracts of this tissue the presence of two photosensitive pigments could be demonstrated. One was ordinary rhodopsin. The other was a new substance which, like the cones themselves, was sensitive to deep red light, to which the rods and rhodopsin scarcely respond. This substance also had absorption properties in the spectrum similar to the spectral sensitivity of cone vision in chickens (27a). It was clearly the photopigment of the cones. Its spectral characteristics showed it to be violet in color. I have therefore called it *iodopsin* (65).

Nothing is yet known directly of the chemical structure of this substance. Recent demonstrations that in human vitamin A deficiency the thresholds of cones as well as rods are increased suggest that human cone photopigments may be derived from vitamin $A_1$ (21, 76). The great similarity in spectral behavior of iodopsin, rhodopsin and porphyropsin implies a close chemical relationship. Indeed the spectra of these three pigments form a progressive series which suggests that the same type of modification in the carotenoid prosthetic group which differentiates rhodopsin and porphyropsin may be extended further to produce iodopsin. In any case it is probable that iodopsin, like its rod counterparts, is a carotenoid-protein.

With the cone photopigment one encounters the problem of color vision. In man, whose color vision is based upon three primaries, it is commonly assumed that the cones contain three photopigments which differ in absorption spectra. In the retina of the chicken, however, as in a number of other birds and certain turtles, an independent basis for color differentiation exists in a tripartite system of colored oil globules. These are situated in the inner limbs of the cones at the junction with the outer limbs, so that light must pass through them before entering the photosensitive segments. They form three distinct groups, red, golden and greenish yellow in color; and it was long ago suggested that they might form an arrangement for color vision comparable to three-filter systems used in color photography.

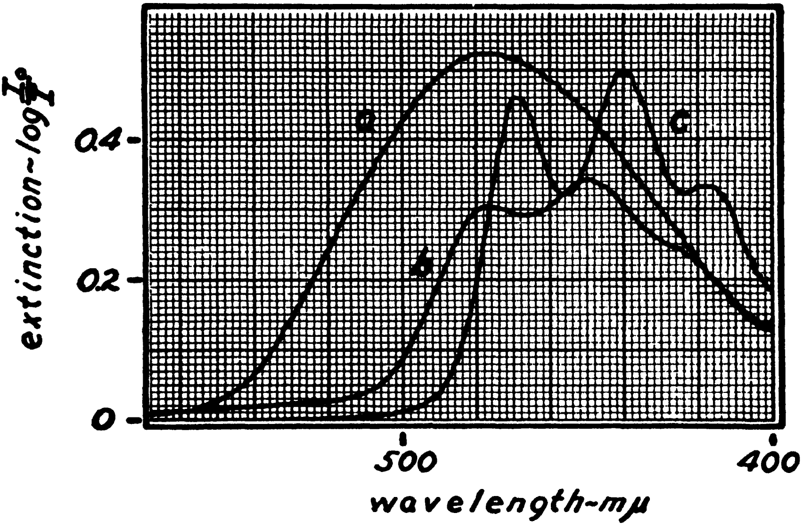


FIG. 12. Filter pigments of the chicken cones. Spectra of crystalline carotenoids from the chicken retina, dissolved in hexane: (a) astacene, the autoxidation product of astaxanthin; (b) xanthophyll, probably a mixture of lutein and zeaxanthin such as occurs in chicken egg yolk; and (c) an unidentified carotene (77).

We have extracted and fractionated the filter pigments of the chicken retina (77). They are all carotenoids: the red astaxanthin, a golden mixture of the plant xanthophylls lutein and zeaxanthin, and a greenish-yellow, unidentified carotene (figure 12). It could be shown that the astaxanthin is synthesized by the chicken itself, for it is absent from the egg yolk, yet it appears in the retina of the embryo during incubation. We were unable to find a trace of astaxanthin in chicken liver, serum, or other tissues, or in the whole bodies of newly hatched chicks less the eyes (77, 74).

One has as yet no insight into the chemistry of human color vision. Certainly it does not depend upon such arrangements of retinal color filters as are found in birds and turtles. Yet in the human retina also one finds a filter pigment, diffusely distributed throughout the region of highest cone concentration.

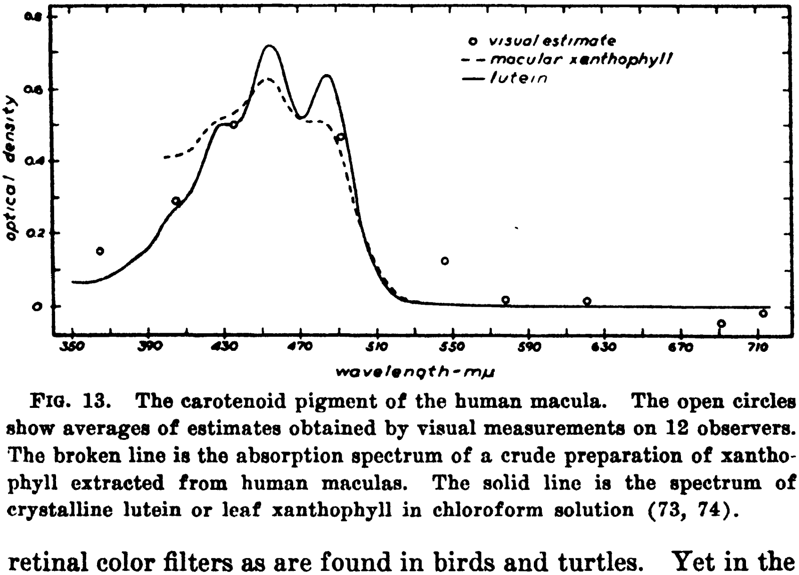


FIG. 13. The carotenoid pigment of the human macula. The open circles show averages of estimates obtained by visual measurements on 12 observers. The broken line is the absorption spectrum of a crude preparation of xanthophyll extracted from human maculas. The solid line is the spectrum of crystalline lutein or leaf xanthophyll in chloroform solution (73, 74).

At the center of fixation in the human retina is a small, shallow pit, the fovea, the floor of which contains only cones. This is the area of most acute vision. For a small distance about it, cones greatly predominate over rods. This entire region is colored yellow, and is therefore called the yellow patch, or *macula lutea*. Such a pigmented central area is a rare phenomenon, found, so far as known, only in primates. Its function, as Max Schultze suggested in 1866, is probably to sharpen the retinal image by excluding the blue and violet regions of the spectrum, for which the eye possesses a large chromatic aberration.

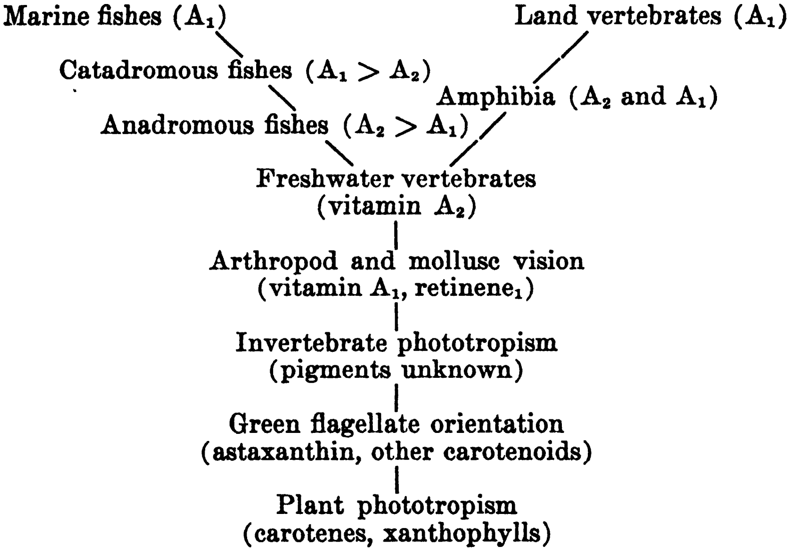
The presence of this yellow filter greatly decreases the sensitivity of the central retina for blue and violet light. By comparing the spectral sensitivity of human cone vision in the macula and in an unpigmented peripheral area, I was able to estimate roughly the absorption spectrum of the macular pigment *in vivo.* Values so obtained are shown as open circles in figure 13. The shape and position of this spectrum suggested that the macular pigment may be a carotenoid. On extracting a small number of human maculas I found that the pigment is in fact a xanthophyll, in all probability lutein or leaf xanthophyll itself (figure 13) (73).

What little is known of the chemistry of cone vision, therefore, seems to show that it depends upon carotenoids not only for the photoreceptor function, but for its accessory pigments. The latter have an oddly reminiscent character. They bring our argument back to the substances with which it began, the carotenoids concerned with photoreception in molds and green organisms. In part this is a natural result of the dependence of animals on plants for their carotenoids. The appearance of leaf xanthophylls in sauropsidan cones and in human maculas can be so explained. In part it involves curiously recurrent syntheses of the zoocarotenoid astaxanthin. This is first encountered in the green flagellates as the principal pigment of the eye-spot; and it is probably only these organisms that can synthesize it *de novo.* It is found again in the eyes, and generally distributed about the integument, of the crustacea. Finally it appears in the cones of birds and turtles as a filter pigment, concerned perhaps with color differentiation; and in the chicken, where it is confined to the retina, it seems to have been elaborated specifically for the cones.

## SUMMARY

I have tried to bring together here the evidence that in plants as in animals carotenoids play a major part in the photoreceptor process; and that this connection provides a molecular homology by means of which one can trace the chemical evolution of vision.

The evidence is reasonably clear that in certain molds and higher plants the light which stimulates bending and other orientation reactions is absorbed by carotenes and xanthophylls. In certain green flagellates, and apparently concentrated in their eye-spots, these pigments reappear in company with a new and predominant carotenoid, astaxanthin, found otherwise only in animal tissues. The chemical basis of photoreception in lower invertebrates is still unexplored. By the time image-forming eyes have appeared in arthropods and molluscs, animals have lost the capacity to synthesize carotenoids *de novo,* and the active role in photoreception has passed to vitamin $A_1$ and retinene$_1$.


Marine fishes ($A_1$)
Land vertebrates ($A_1$)
Catadromous fishes ($A_1 > A_2$)
Amphibia ($A_2$ and $A_1$)
Anadromous fishes ($A_2 > A_1$)
Freshwater vertebrates
(vitamin $A_2$)
Arthropod and mollusc vision
(vitamin $A_1$, retinene$_1$)
Invertebrate phototropism
(pigments unknown)
Green flagellate orientation
(astaxanthin, other carotenoids)
Plant phototropism
(carotenes, xanthophylls)

The emergence of freshwater vertebrates introduces the porphyropsin system of the rods, based upon retinene$_2$ and vitamin $A_2$. This thereafter remains genetically associated with freshwater existence, and particularly with the habit of spawning in fresh water. Vertebrate evolution has followed two pathways out of fresh water, one into the sea, the other to land. Both developments have led it back to the use of vitamin $A_1$, and to the rhodopsin system.

Interpolated between freshwater and marine fishes are euryhaline forms which as adults can tolerate both environments. They possess both the rhodopsin and porphyropsin systems, frequently in mixtures, and always predominantly the system appropriate to the environment in which the fish spawns. Similarly interpolated between freshwater fishes and land vertebrates are the amphibia, in which again both systems appear. The bullfrog during metamorphosis transfers from the porphyropsin to the rhodopsin system, and so seems to recapitulate the evolutionary change which marked the emergence of land vertebrates.

These relations are summarized in the diagram on page 157.

In vertebrate cones a new photosensitve pigment, iodopsion, is found. Its composition is not known, but indirect evidence indicates that it is a carotenoid-protein, closely related to the rod photopigments. In the cones of birds and turtles colored globules appear which may serve the function of color differentiation; from these the carotenoids astaxanthin, plant xanthophylls, and a carotene have been isolated. The area in which cones are most concentrated in the human retina is colored yellow, and this pigment also has been identified as a xanthophyll, probably lutein or leaf xanthophyll itself. Cone vision, therefore, like that of the rods, seems to depend upon vitamin A-like substances in photoreception; and tends to meet its accessory needs with such intact carotenoids as principally mediate photoreception in plants and green flagellates.

## REFERENCES

1. Bachmann, F., and Bergann, F., *Planta,* 1930, 10, 744.
2. Baldwin, E., and Needham, D. M., *Proc. Roy. Soc.* London, B, 1937, 122, 197.
3. Blaauw, A. H., *Rec. Trav. bot. néerl.,* 1909, 5, 209.
4. Bliss, A. F., *J. Gen. Physiol.,* 1942–43, 26, 361.
5. Boll, Franz, *Arch. f. Physiol.,* 1877, 4.
6. Bottelier, H. P., *Proc. Kon. Akad. Wetensch.* Amsterdam, 1933, 36, 3.
7. Buder, J., *Beitr. Biol. Pflanz.,* 1932, 19, 420.
8. Bünning, E., *Planta,* 1937, 26, 719.

9. Bünning, E., *Planta*, 1937, **27**, 148
10. Bünning, E., *Planta*, 1937, **27**, 583.
11. Castle, E. S., *J. Gen. Physiol.*, 1931, **14**, 701.
12. Castle, E. S., *Cold Spring Harbor Symp.*, 1935, **3**, 224.
13. Crowell, M. F., and McCay, C. M., *Physiol. Zool.*, 1937, **10**, 368.
14. Drummond, J. C., and Gunther, E. R., *J. Exp. Biol.*, 1934, **11**, 203.
15. Dutton, H. J., and Manning, W. M., *Am. J. Bot.*, 1941, **28**, 516.
16. Edisbury, J. R., Morton, R. A., and Simpkins, G. W., *Nature*, 1937, **140**, 234.
17. Emerson, R., and Lewis, C. M., *J. Gen. Physiol.*, 1941–42, **25**, 579.
18. Engelmann, T. W., *Arch. ges. Physiol.*, 1882, **29**, 387.
19. Escher-Desrivières, J., Lederer, E., and Verrier, M.-L., *C. R. Acad. Sci. Paris*, 1938, **207**, 1447.
20. Gillam, A. E., El Ridi, M. S., and Wimpenny, R. S., *J. Exp. Biol.*, 1939, **16**, 71.
20a. Gillam, A. E., Heilbron, I. M., Jones, W. E., and Lederer, E., *Biochem. J.*, 1938, **32**, 405.
21. Haig, C., Hecht, S., and Patek, A. J., Jr., *Science*, 1938, **87**, 534.
22. Hall, F. G., *J. Physiol.*, 1934, **82**, 33.
23. Hall F. G., *J. Physiol.*, 1934–35, **83**, 222.
24. Hartline, H. K., *Harvey Lectures*, 1941–42, **37**, 39.
25. Hecht, S., *J. Gen. Physiol.*, 1920–21, **3**, 375.
26. Hecht, S., *J. Gen. Physiol.*, 1927–28, **11**, 657.
27. Hecht, S., *Harvey Lectures*, 1937–38, **33**, 35.
27a. Honigmann, H., *Arch. ges. Physiol.*, 1921, **189**, 1.
28. Hunter, R. F., and Hawkins, E. G. E., *Nature*, 1944, **153**, 194.
29. Johnston, E. S., *Smithsonian Misc. Publ.*, 1934, **92**, No. 11.
30. Karrer, P., and Bretscher, E., *Helv. Chim. Acta*, 1943, **26**, 1758.
31. Karrer, P., and Jaffé, W., *Helv. Chim. Acta*, 1939, **22**, 69.
32. Köttgen, E., and Abelsdorff, G., *Z. Psychol. u. Physiol. Sinnesorgane*, 1896, **12**, 161.
33. Kuhn, R., *Angew. Chemie*, 1937, **50**, 703.
34. Kuhn, R., and Sörensen, N. A., *Ber. deutsch. chem. Ges.*, 1938, **71**, 1879.
35. Kuhn, R., Stene, J., and Sörensen, N. A., *Ber. deutsch chem. Ges.*, 1939, **72**, 1688.
36. Kühne, W., Chemische Vorgänge in der Netzhaut, in Hermann's Handbuch der Physiologie, Leipzig, 1879, v. 3, part 1, p. 235.
37. Kühne, W., and Sewall, H., *Untersuch. physiol. Inst. Univ. Heidelberg*, 1880, **3**, 221.
38. Laurens, H., and Hooker, H. D., Jr., *J. Exp. Zool.*, 1920, **30**, 345.
39. Lederer, E., Rosanova, V., Gillam, A. E., and Heilbron, I. M., *Nature*, 1937, **140**, 233.
40. Loeb, J., *Arch. ges. Physiol.*, 1890, **47**, 391.

41. Loeb, J., and Wasteneys, H., *J. Exp. Zool.*, 1915, **19**, 23.
42. Luntz, A., *Z. vergl. Physiol.*, 1931, **14**, 68.
43. Luther, R., and Plotnikow, J., *Z. physikal. Chem.*, 1908, **61**, 513.
44. Luther, R., and Weigert, F., *Z. physikal. Chem.*, 1905, **53**, 385.
45. Mast, S. O., *J. Exp. Zool.*, 1917, **22**, 471.
46. Mast, S. O., *Arch. Protistenk.*, 1927, **60**, 197.
47. McCay, C. M., *Physiol. Zool.*, 1938, **11**, 89.
48. McCutcheon, F. H., *J. Cel. Comp. Physiol.*, 1936, **8**, 63.
49. Meyerhof, O., Chemische Vorgänge im Muskel, Berlin, J. Springer, 1930 (p. 93).
50. Morton, R. A., and Goodwin, T. W., *Nature*, 1944, **153**, 405.
51. Needham, D. M., Needham, J., Baldwin, E., and Yudkin, J., *Proc. Roy. Soc.* London, B, 1932, **110**, 260.
52. Redfield, A. C., *Amer. Nat.*, 1936, **70**, 110.
53. Romer, A. S., and Grove, B. H., *Amer. Midland Nat.*, 1935, **16**, 805.
54. Sachs, J., *Botan. Ztg.*, 1864, **22**, 353, 361, 369.
55. Schopfer, W.-H., *C. R. Soc. Biol.*, 1935, **118**, 3.
56. Smith, H. W., *Quart. Rev. Biol.*, 1932, **7**, 1.
57. Stern, K. G., and Salomon, K., *J. Biol. Chem.*, 1938, **122**, 461.
58. Tischer, J., *Z. physiol. Chem.*, 1936, **239**, 257.
59. Tischer, J., *Z. physiol. Chem.*, 1938, **252**, 225.
60. Voerkel, S. H., *Planta*, 1933, **21**, 156.
61. Wald, G., *J. Gen. Physiol.*, 1935–36, **19**, 351.
62. Wald, G., *J. Gen. Physiol.*, 1935–36, **19**, 781.
63. Wald, G., *J. Gen. Physiol.*, 1936–37, **20**, 45.
64. Wald, G., *Nature*, 1937, **139**, 1017.
65. Wald, G., *Nature*, 1937, **140**, 545.
66. Wald, G., *J. Gen. Physiol.*, 1937–38, **21**, 795.
67. Wald, G., *J. Gen. Physiol.*, 1938–39, **22**, 391.
68. Wald, G., *J. Gen. Physiol.*, 1938–39, **22**, 775.
69. Wald, G., *Am. J. Physiol.*, 1941, **133**, 479.
70. Wald, G., *J. Gen. Physiol.*, 1941–42, **25**, 235.
71. Wald, G., *J. Gen. Physiol.*, 1941–42, **25**, 331.
71a. Wald, G., *Biol. Symp.*, 1942, **7**, 43.
72. Wald, G., *Vitamins and Hormones*, 1943, **1**, 195.
73. Wald, G., *Science*, 1945, **101**, 653.
74. Wald, G., Unpublished observations.
75. Wald, G., and du Buy, H. G., *Science*, 1936, **84**, 247.
76. Wald, G., Jeghers, H., and Arminio, J., *Am. J. Physiol.*, 1938, **123**, 732.
77. Wald, G., and Zussman, H., *J. Biol. Chem.*, 1938, **122**, 449.

# EXPERIMENTS WITH BACTERIAL VIRUSES (BACTERIOPHAGES)[1]

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THE history of research on bacterial viruses or bacteriophages, now 30 years old, is fraught with controversy. The puzzling nature of these agents, living or inanimate, enzyme or virus, and the alluring possibilities of their useful application in medical practice have in times past attracted great scientists who spent their best efforts and keen imagination on devising ever new avenues of approach. This heroic age seems now a matter of the past, the passions have subsided and minor men with different interests are beginning to settle on these grounds. These minor men of our present age come from such far outlying fields as physics, genetics, biochemistry. They have a feeling that today many lines of biological research are converging on a central problem, the organization of the cell, a feeling similar to that which inspired the physicists of 50 years ago, when the structure of matter and the constitution of the atoms became the focus on which all efforts converged. They feel that the field of bacterial viruses is a fine playground for serious children who ask ambitious questions. You might wonder how such naïve outsiders get to know about the existence of bacterial viruses. Quite by accident, I assure you. Let me illustrate by reference to an imaginary theoretical physicist, who knew little about biology in general, and nothing about bacterial viruses in particular, and who accidentally was brought into contact with this field. Let us assume that this imaginary physicist was a student of Niels Bohr, a teacher deeply familiar with the fundamental problems of biology, through tradition, as it were, he being the son of a distinguished physiologist, Christian Bohr.

Suppose now that our imaginary physicist, the student of Niels Bohr, is shown an experiment in which a virus particle enters a bacterial cell and 20 minutes later the bacterial cell is lysed and 100 virus particles are liberated. He will say: "How come, one particle has become 100 particles of the same kind in 20 minutes? That is very interesting. Let us find out how it happens! How does the particle get in to the bacterium? How does it multiply? Does it multiply like a bacterium, growing and dividing, or does it multiply by an entirely different mechanism? Does it have to be inside the bacterium to do this multiplying, or can we squash the bacterium and have the multiplication go on as before? Is this multiplying a trick of organic chemistry which the organic chemists have not yet discovered? Let us find out. This is so simple a phenomenon that the answers cannot be hard to find. In a few months we will know. All we have to do is to study how conditions will influence the multiplication. We will do a few experiments at different temperatures, in different media, with different viruses, and we will know. Perhaps we may have to break into the bacteria at intermediate stages between infection and lysis. Anyhow, the experiments only take a few hours each, so the whole problem can not take long to solve."

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[1] Lecture delivered on January 17, 1946.

Perhaps you would like to see this childish young man after eight years, and ask him, just offhand, whether he has solved the riddle of life yet? This will embarrass him, as he has not got anywhere in solving the problem he set out to solve. But being quick to rationalize his failure, this is what he may answer, if he is pressed for an answer: "Well, I made a slight mistake. I could not do it in a few months. Perhaps it will take a few decades, and perhaps it will take the help of a few dozen other people. But listen to what I have found, perhaps you will be interested to join me."

Remember that what we are out to study is the multiplication process proper, we want to get to the bottom of what goes on when more virus particles are produced upon the introduction of one virus particle into a bacterial cell. All our work has circled around this central problem. Of course if anybody wants to be disagreeable at this point he can quarrel with the very statement of the problem. He might question whether really more virus particles are produced, perhaps the apparently produced ones were already in the cell and make their appearance because they become released. Or he might question whether one really "introduces" a virus particle into the bacterial cell, perhaps the virus particle sticks to the outside, and stimulates a process within the cell, or on the surface of the cell. We will overlook these doubts for the time being; perhaps they will disappear after we have looked at a few experiments.

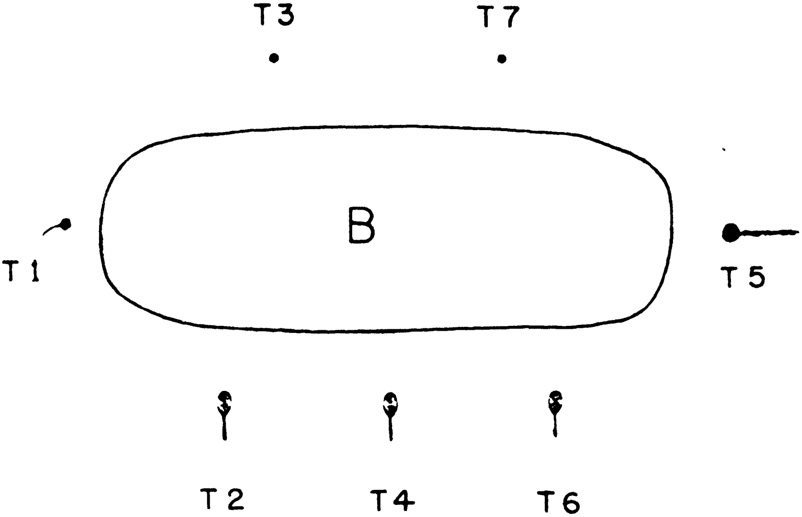

FIG. 1. Relative sizes of the E. coli strain *B* (the bacterial host) and the seven virus strains used in these studies. The viruses of like shape are also serologically related.

It follows from the nature of our approach that we plan to be impartial, taking sides neither with the viruses nor with the bacteria. We are not primarily interested in the destruction of the *bacteria*, intent on applying what we find to the therapy of infectious diseases caused by *bacteria*. Nor are we interested, primarily, in devising means to frustrate the growth of the *viruses*, intent on applying such knowledge to the therapy of infectious diseases in plants, animals and men caused by *viruses*. Such motives, noble though they are, are ulterior to our cause.

First of all I would like to introduce you to the material with which most of our work has been concerned.[2] It consists of one strain of Escherichia coli, called strain *B*, and of seven strains of bacterial viruses, called T1, T2, . . . and T7. The sizes and shapes of these viruses are approximately known from electron micrographs. They are represented in figure 1 in the proper size relationship with their host bacterium *B*. You will note that the even numbered viruses T2, T4, and T6 look alike. All three have a peculiar head structure and a straight tail. These three virus strains are also serologically related. T3 and T7 are the smallest members of the family. They show no tails and no head structure. Specific antisera to these two viruses show cross reactions, but only at high concentration of the sera. Both T1 and T5 stand serologically and morphologically by themselves.

This family of viruses has developed by accretion. As work progressed gradually more viruses were included in our studies. It would be easy to enlarge this family still further but our tendency has been to keep the family small, so that every member can get a maximum of attention.

Since we wish to analyze the multiplication of a virus within the cell of its bacterial host it should be our first aim to develop a method of determining the number of virus particles which are present within a bacterial cell at any one moment. Here I, and those who have been associated with me in this work, have to make the first admission of failure. Neither we nor anybody else has yet succeeded in devising a method that would allow us to count the number of virus particles within a cell before lysis. Some attempts have been made to break open infected cells at intermediate stages, or to apply indirect methods for the determination of the number of intracellular virus particles. The results of such attempts were either completely negative or the interpretation of the results was ambiguous. It is quite likely that future attempts will be more successful.

Since we could not count the number of intracellular virus particles, what then can we count? Essentially two things.

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[2] A more detailed description of this material may be found in (1).

First, we can count the number of extracellular, or "free" virus particles, second we can count the number of infected bacteria. Both of these numbers are determined by plaque counts. In a growing mixture of bacteria and virus, containing both free virus and infected bacteria a plaque count will determine the *sum* of these two quantities. One can eliminate either the free virus or the infected bacteria and determine the plaque count for the other alone. The free virus can be eliminated by specific antiserum, which does not inactivate infected bacteria (2). On the other hand, the infected bacteria can be eliminated by centrifuging them down. The assay of the supernatant will then be an assay of the free virus alone.

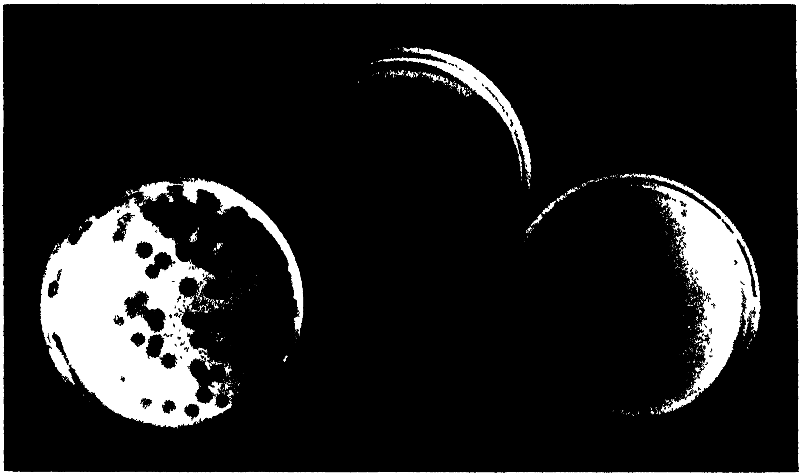

FIG. 2. Platings of a growing mixture of *B* and T7. *Left:* Total infected *B* and free T7. *Center:* Infected *B* (free T7 eliminated with antiserum). *Right:* Free T7 (infected *B* centrifuged out).

Figure 2 illustrates these points. It shows three plates, on which equal samples of the same growing mixture of bacteria and virus were plated. The first plate is a plating of the untreated mixture. A plaque on this plate may be due either to a free virus particle or to an infected bacterium. The second plate is a plating from the same mixture after the bacteria were centrifuged out. The plaques on this plate are due to free virus particles exclusively. The third plate is a plating from the same mixture after specific antivirus serum had been added and permitted to act for four minutes. The plaques on this plate are due to infected bacteria exclusively. It will be seen that the plaque counts on the second and third plate add up, within the limits of sampling errors, to the count on the first plate.

I wish to point out in passing that these plates were prepared by the agar-layer method, first described by Gratia (3), which has recently come into wider use. In this technique (figure 3) a few milliliters of melted agar of low concentration, containing the bacteria, are mixed with the sample to be plated. This mixture is poured on the surface of an ordinary nutrient agar plate. The mixture distributes itself uniformly in a very thin layer over the plate and solidifies immediately. This method combines the virtues of two methods previously employed, viz., the deep agar method and the surface spreading method.

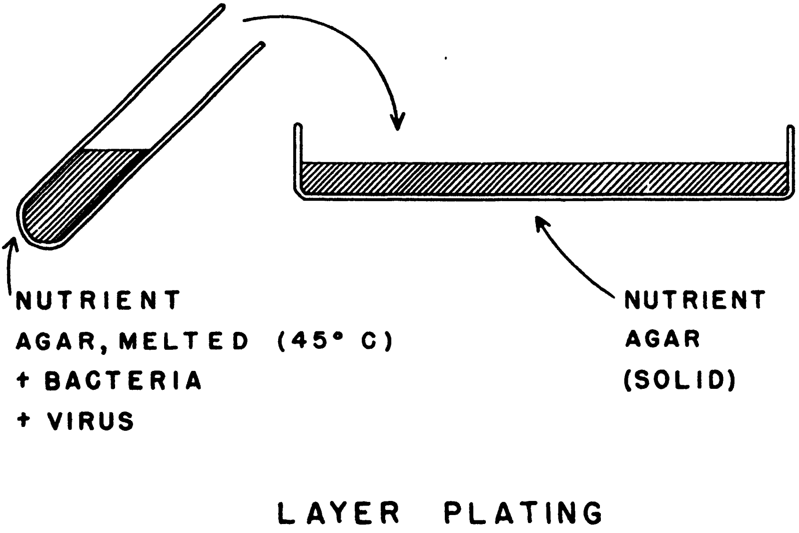

FIG. 3. Agar-layer technique for plating.

With this understanding about the meaning and the limitations of plaque count assays, how can we use plaque counts for the study of virus multiplication? The plaque count obtainable from a growing mixture of bacteria and viruses will change either when the number of free virus particles changes or when the number of infected bacteria changes. The number of free virus particles will decrease when a virus particle becomes attached to a bacterium. This process will not change the total plaque count since the free virus particle is replaced by an infected bacterium.

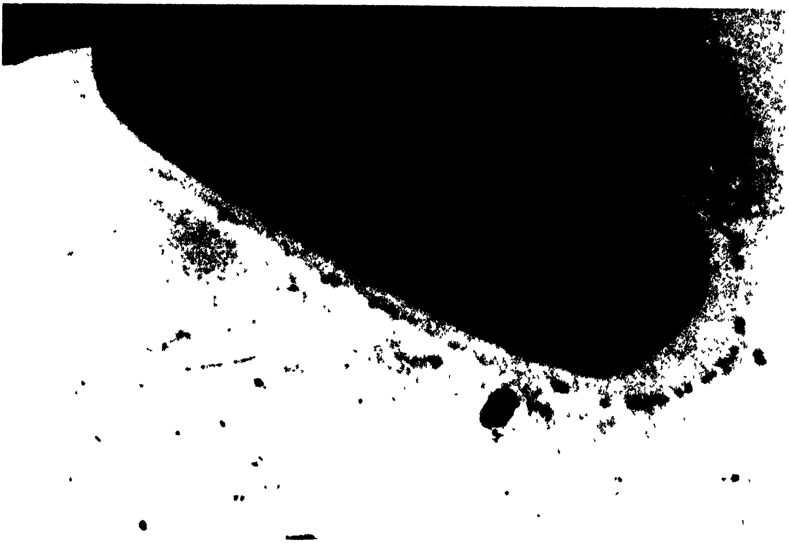

FIG. 4. Action shot of a bacterium lysed by virus T3. The specimen was dried 17 minutes after mixing virus and bacteria.

An exception occurs when the bacterium to which the virus particle attaches itself was already infected. In that case the number of infected bacteria is not increased by the second infection and we have a net loss of one plaque former. This contingency arises when an excess of virus particles is mixed with bacteria. Each bacterium will be "multiply infected" and a net loss of plaque formers occurs.

The number of free virus particles increases when an infected bacterium releases its virus content. This release occurs with out. The plaques on this plate are due to free virus particles exclusively. The third plate is a plating from the same mixture after specific antivirus serum had been added and permitted to act for four minutes. The plaques on this plate are due to infected bacteria exclusively. It will be seen that the plaque counts on the second and third plate add up, within the limits of sampling errors, to the count on the first plate.

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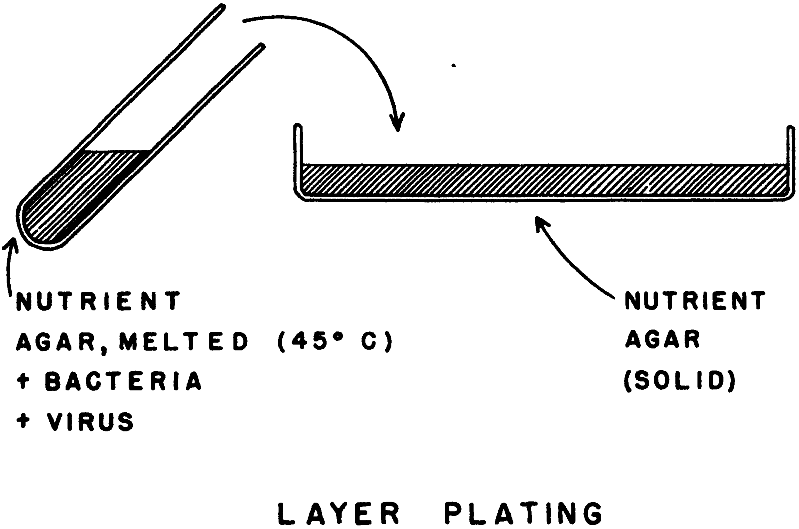

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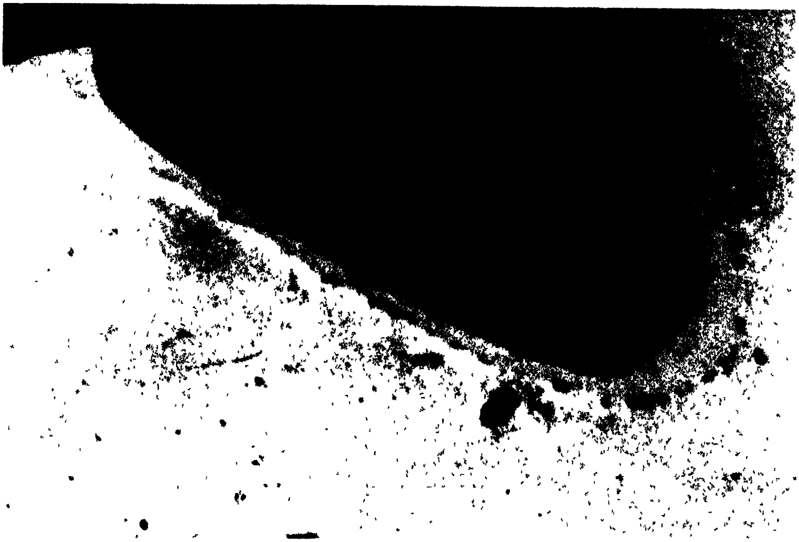

FIG. 4. Action shot of a bacterium lysed by virus T3. The specimen was dried 17 minutes after mixing virus and bacteria.

The number of free virus particles increases when an infected bacterium releases its virus content. This release occurs with dramatic suddenness when the bacterium is lysed. I should like to insert at this point an electron microscope picture[3] which represents the nearest approach to action shots of this process (figure 4). Time does not permit to discuss other features revealed by electron microscope pictures (4, 4a). I only wish to draw attention to the evidence given by this picture for the fact that virus release occurs at the moment of lysis.

The release of, say, one hundred virus particles upon lysis of the bacterium means a corresponding increase in the plaque count of free virus particles, and a decrease, but only by one, of the plaque count of infected bacteria. The net effect, therefore, is a large increase in the plaque count.

Figure 5 represents an experiment in which virus T1 was added to a growing culture of bacteria of strain *B* at time zero (5). The bacteria were in excess so that not all bacteria were infected and those that were infected received in most instances only one virus particle. Samples were taken from this mixture every few minutes, and were plated for plaque count after suitable dilution. The plaque count stays constant for 13 minutes. This constancy of the plaque count means that up to 13 minutes no virus particles were released.

After 13 minutes the plaque count begins to rise sharply and in a few minutes attains a level about 60 times the original level. This increase is due to the release of newly formed virus particles from lysing bacteria.

After the initially infected bacteria have all been lysed the plaque count levels off. After the lapse of another 13 minutes another rise of the plaque count might be expected from bacteria that were infected with virus particles released in the first step. In experiments of this kind we obviate this re-infection by diluting the growing mixture a few minutes after it has been set up. The dilution effectively stops adsorption. In this way the events connected with the first step are brought out more clearly. In particular, the minimum latent period, in this case 13 minutes, and the average yield of virus per bacterium can be measured with fair accuracy. The minimum latent period is read from such a graph as here shown. The average yield of virus per bacterium is obtained from the size of the step in this curve. The upper level represents the total yield of virus particles liberated from the infected bacteria. To obtain the average yield per infected bacterium we must divide the number of infected bacteria into this total yield. The number of infected bacteria is determined, for instance, by a plaque count, before the onset of lysis, of a sample of the culture in which the free virus has been eliminated by specific antiserum.

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[3] This picture was taken by the author in collaboration with Dr. E. G. Pickels, of the New York laboratories of the International Health Division of The Rockefeller Foundation.

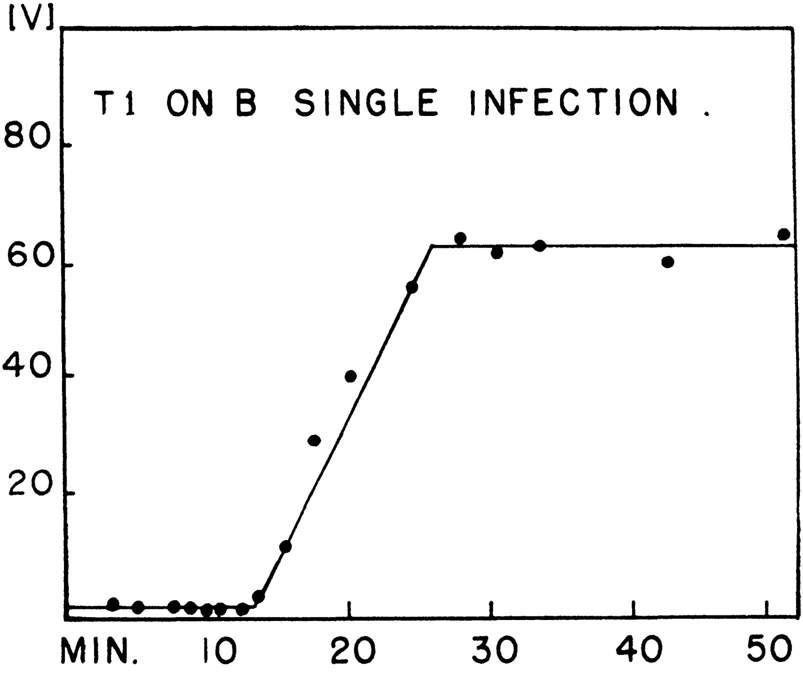

FIG. 5. One step growth curve, virus T1, single infection.

An experiment of this type is called a one-step growth experiment (6). Before proceeding with the discussion of similar experiments I would like to fill in a gap. We have assumed, following d'Herelle, that virus liberation occurs when, and only when, the corresponding host bacterium is lysed. This assumption is supported by direct electron microscope evidence. It is also supported by the fact that lysis of the bacteria, as seen under the ordinary microscope, occurs approximately at the right time, i.e., at the time corresponding to the rise of plaque count in the one-step growth curve. This agreement between the time of onset of lysis and of onset of plaque count increase can be verified more accurately by measuring the turbidity of cultures to which an excess of virus is added at time zero.

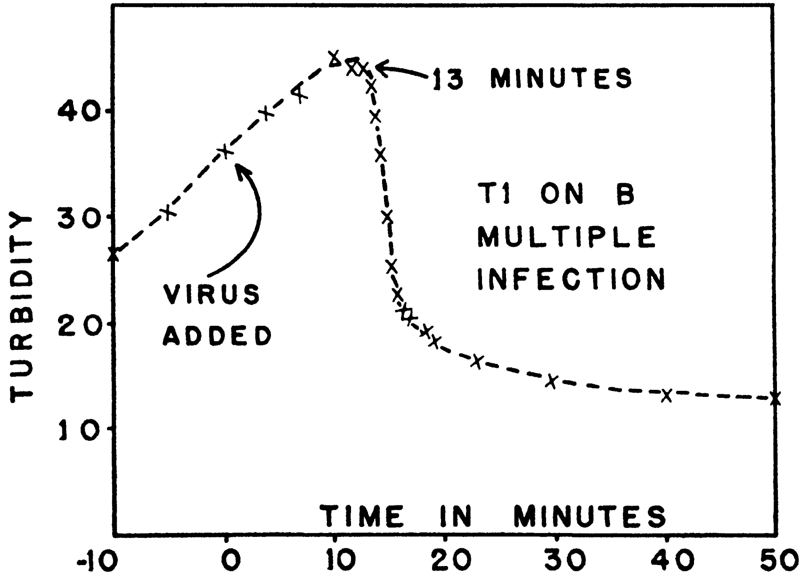

FIG. 6. Turbidity changes in a culture of *B*, multiply infected with virus T1.

Figure 6 shows an experiment in which the turbidity was measured with a photoelectric nephelometer constructed by Dr. N. Underwood of the Physics Dept. of Vanderbilt University. It will be seen that the turbidity begins to drop exactly 13 minutes after virus was added. You will recall from the preceding slide that in the one-step growth experiment the plaque count began to rise also exactly 13 minutes after virus was added.

The conditions in these two experiments differed in one respect. In the one-step growth experiment the number of virus particles added to the culture was smaller than the number of bacteria, while in the turbidity experiment the number of virus particles added at time zero was about five times greater than the number of bacteria. Each bacterium then picks up several virus particles. One might expect that this would shorten the minimum latent period for virus liberation. Surprisingly enough experiments show that this is not so. The figure[4] shows two one-step growth experiments with single and multiple infection, respectively, of *B* by the virus T2. The minimum latent period is in both cases 21 minutes. This equality of the minimum latent period for single and multiple infection has been found true for all viruses studied. The minimum latent period differs greatly from one virus strain to another, but for any given virus strain the minimum latent period is independent of the multiplicity of infection.

For the seven viruses which have been used in these studies the minimum latent period is shortest (13 minutes) for T1 and T7, and longest (40 minutes) for T5.

These minimum latent periods were obtained in experiments run at a temperature of 37° C. At other temperatures the minimum latent periods differ from these values roughly in proportion to the degree to which these other temperatures affect the rate of growth of the bacteria (6).

The parallelism between the minimum latent period and the growth rate of the bacteria breaks down when different types of nutrient media are used. Thus, the minimum latent periods of T1, T2, and T7 are the same in synthetic media as in broth, although the bacteria grow much more slowly in the synthetic media than in broth (1). One infers that the chain of processes which leads to the lysis of the bacteria is tied to some branch of the bacterial metabolism whose rate is not altered in the synthetic media. What branch of the bacterial metabolism this may be is at present unknown. Attempts to identify this branch (by the study of the effects of a variety of substrates and of metabolic inhibitors on one-step growth experiments) have not so far yielded significant results. There can be no doubt, however, that further studies along these lines constitute one of the most promising lines of attack.

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[4] See Fig. 3 of reference (5).

At this point I would like to mention an extension of the technique of one-step growth experiments which enables one to study the yields from individual bacteria. This method was invented long ago by Burnet (7). In order to separate the yields from individual bacteria one has to segregate the bacteria before they lyse. In Burnet's method this is done simply by a suitable dilution procedure, illustrated in figure 7. At time zero bacteria and virus particles are mixed. After five minutes the majority of the virus particles have become attached to bacteria and have begun

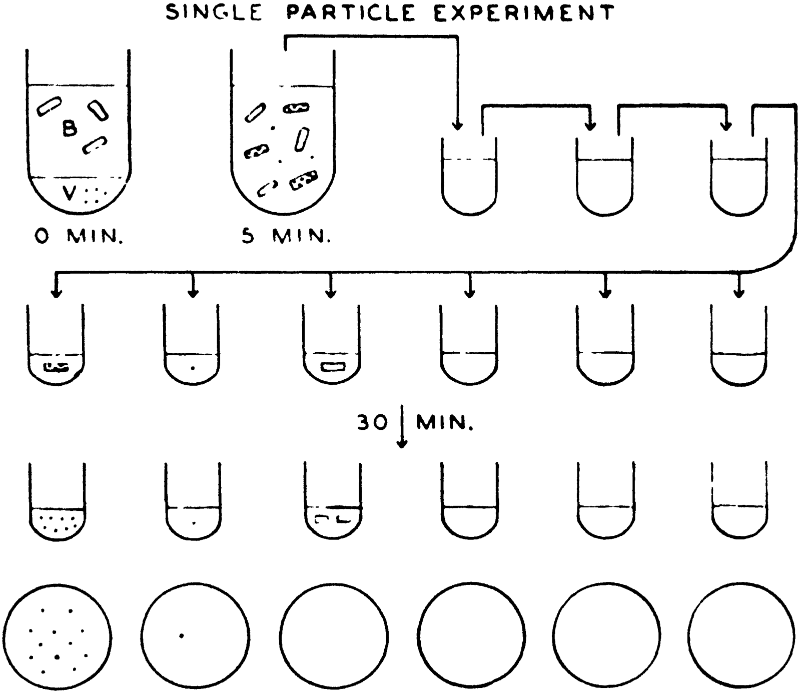

FIG. 7. Procedure for recording the yields from individual bacteria.

to multiply within the bacteria. The mixture is then rapidly diluted in several steps (three steps in the illustration) so that in the last of these dilution tubes there is an average only of one-half infected bacterium per drop. Drops from this tube are then placed into numerous small tubes, one drop into each tube. This operation must be completed before the onset of lysis. We have then in the majority of these tubes neither virus particles nor bacteria. In some there will be an infected bacterium, in some a free virus particle, and in some an uninfected bacterium. If these tubes are incubated until after lysis time, and then plated for plaque count, the tubes without virus particles will give no plaque, while those with an infected bacterium will give numerous plaques, one for each of the virus particles released from this particular bacterium. In this manner the yields from individual bacteria can be measured. There are certain obvious limitations of a statistical nature in this method into which I will not go here.

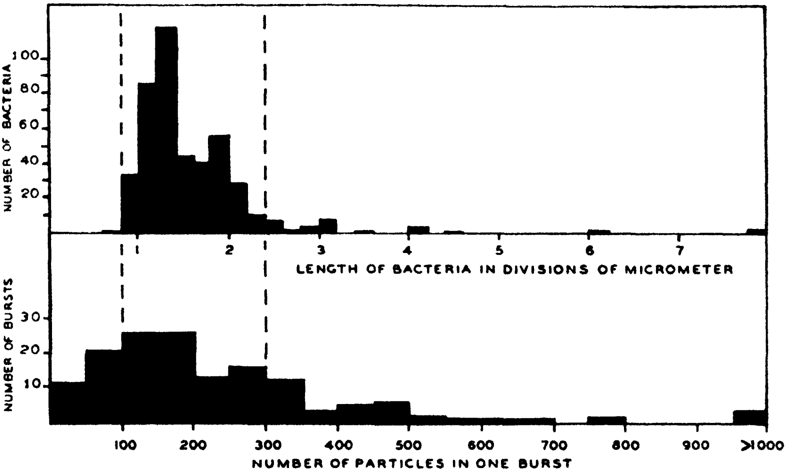

FIG. 8. Distribution of burst sizes (lower half) and of bacterial sizes (upper half). Virus T1, single infection.

The application of this method to a variety of bacterial viruses has given an unexpected result (8). It turns out that the yield of a given virus from individual bacteria shows very wide variations (See figure 8). Of course a variation in yield by a factor two is to be expected since the bacteria in these experiments are growing and dividing, and, therefore, must vary in size at least in a range of a factor two. Microscopic examination showed that the bacterial sizes vary by just about this amount. The wide variation in the yield of virus can therefore not be accounted for by variation in bacterial size alone. The interpretation of this wide variation at present could only be speculative and will therefore not be attempted.

This method for studying the yields from individual bacteria has also been used in the study of bacteria mixedly infected with particles belonging to two different strains of virus.

When Dr. Luria and I in 1941 (5) began doing experiments on mixed infection our hope was that the bacteria would be lysed after a time interval corresponding to the shorter of the latent periods of the two viruses, and that the yield of the other virus would reflect an intermediate stage in the multiplication of this virus. In other words, we were hoping that the two viruses would multiply within the bacterium independently of each other, in such a manner that the multiplication of one virus was not interfering with the multiplication of the other virus. It turned out, however, that, on the contrary, the two viruses interfere strongly with each other's multiplication. Our findings may have a bearing on interference phenomena observed with animal and plant viruses. At the present moment, however, neither our studies nor the studies on interference in animal (9–14) and plant (15) viruses are sufficiently advanced to warrant a comparison of the details of these phenomena.

I will first indicate briefly the techniques used in interference experiments. Of primary importance is the possibility to assay for each of the viruses separately in mixtures of both strains. This can be done either by the use of indicator hosts or by the use of specific antisera.

Figure 9 shows the application of indicator strains. A mixture of the two viruses T5 and T7 is plated on three plates. In the center plate the strain *B*, which is sensitive to both viruses, is used as host. The plate shows two types of plaques, large ones, due to T7, and small ones, due to T5. On the left plate the same mixture was plated on *B*/7, that is, on a variant strain of *B* which is resistant to T7. It shows only the plaques due to T5, and the number of plaques due to T5 is equal to the number of T5 plaques formed on *B*. On the right plate again the same mixture was plated, on *B*/5, a mutant resistant to T5 but fully sensitive to T7, as can be seen from the fact that there are no T5 plaques and that the number of T7 plaques formed on this plate is the same as on the center plate.

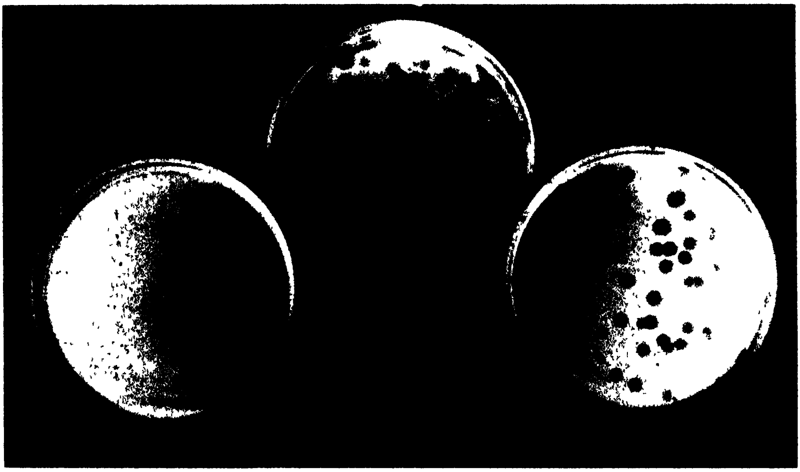

FIG. 9. The use of indicator strains for obtaining separate plaque counts for each of two viruses when plating a mixture of the two viruses. *Left:* T5 on *B*/7. *Center:* T5 and T7 on *B*. *Right:* T7 on *B*/5.

Figure 10 shows the application of specific antisera. The center plate is the same as in the preceding figure. The plate on the left was obtained by plating the same mixture of viruses, on the same host, after exposure of the mixture for three minutes to a 1:50 dilution of specific anti-T7 serum. Similarly the plate on the right was plated after application of anti-T5 serum. The results are similar to those obtained with indicator strains.

The platings shown in the last two figures, it must be understood, were made with *free virus particles*. If, instead of assaying a mixture of free virus particles we had assayed a mixture of *bacteria* of strain *B*, some infected with T5 and some with T7, the two methods would have given quite different results. The method of specific antisera would not work at all, because the anti-serum does not inactivate virus after it has become attached to its host (2). On the other hand, the method of indicator strains will give the same clear-cut segregation for infected bacteria as it does for free virus particles. For this reason the method of indicator strains is of more importance in the study of interference of bacterial viruses than is the use of specific antisera. Specific antisera are of use only when it is desired to quickly eliminate free virus.

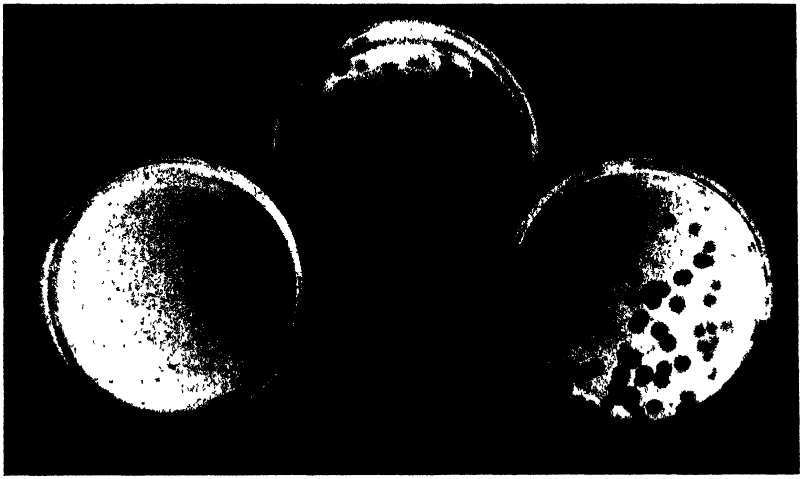

FIG. 10. The use of specific antivirus sera for obtaining separate plaque counts for each of two viruses when plating a mixture of the two viruses. *Center:* Untreated T5 and T7. *Left:* T5 (3 minutes treatment with anti-T7). *Right:* T7 (3 minutes treatment with anti-T5).

Indicator strains can also be used for a very special stunt (16). They can be used to detect whether one and the same bacterium has liberated, upon lysis, both types of virus. This is done by plating mixedly infected bacteria on a mixture of the two corresponding indicator strains. Any plaque that contains only one kind of virus will be turbid since it will be overgrown by the indicator strain which is resistant to this virus. Where plaques of different type overlap, a clear area is formed. Figure 11 shows such accidental overlaps, on one side between two large plaque formers, T1 and T7, on the other side between a large plaque former, T3, and a small plaque former, T5. You will realize that the area of overlap would be strictly concentric if the two viruses were liberated from the same bacterium. A mixture of two indicator strains can therefore be looked upon as an indicator for the *simultaneous* presence of two virus strains in one plaque.

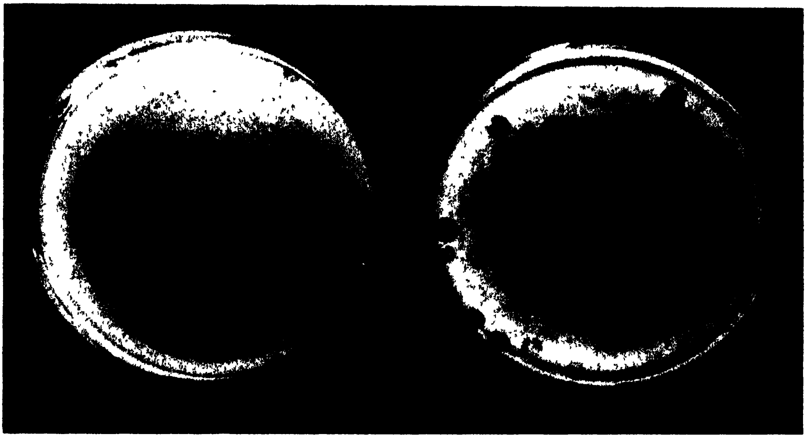

FIG. 11. Plating of mixtures of two viruses with a mixture of two corresponding indicator strains. Only the areas of overlap of plaques of two different types are clear.

Let us look now at the results of experiments in which the bacteria were simultaneously infected with two different viruses. The results may be classified under two headings, namely, "*mutual exclusion*," and "*yield effects*."

***Mutual exclusion*** means that any one mixedly infected bacterium will liberate upon lysis only particles of one of the infecting types. In the case first studied, viruses T1 and T2 were used (6). Here T2 wins out in every case, except when infection with virus T1 precedes infection by virus T2 by more than 4 minutes.

Lysis of the bacteria occurs after 21 minutes, a time interval characteristic for the virus which is liberated. The supremacy of virus T2 over virus T1 persists even if virus T2 is inactivated by the action of ultraviolet light (17). The inactivated virus is unable to reproduce itself but it is still able to exclude virus T1 from reproduction. One has to imagine that the inactive virus makes an abortive attempt at reproduction in the bacterium. This abortive attempt leads to the death of the bacterium and makes the bacterium unfit to serve as host for the reproduction of T1.

The next pair of viruses studied was the pair T1, T7 (16). The members of this pair, as the members of the preceding pair, are serologically and morphologically unrelated Here mutual exclusion is still perfect, although neither of the two viruses is dominant over the other. Every mixedly infected bacterium is lysed and there is a clean split between those bacteria which liberate T1 and those that liberate T7

About a dozen other pairs of viruses have since been tested for the validity of this mutual exclusion principle. In most of these cases the principle appears to hold perfectly. In one or two cases the evidence is not quite conclusive, but in these cases there are other complications, to be referred to presently. Final judgment on these cases must, therefore, be deferred.

There are *yield effects* of several kinds The first kind (16) may be called the "depressor effect," and may be illustrated by the case T1, T7. Here, as pointed out above, the mutual exclusion mechanism works perfectly. Each bacterium liberates either T1 or T7. However, the bacteria which liberate T1 liberate on the average much less T1 than they would have liberated if they had been infected with this virus alone. It is clear that virus T7, while itself unable to multiply in this bacterium, is yet able to interfere with the multiplication of virus T1 and to "depress" the yield of this virus. The same is true, vice versa, for bacteria liberating T7. Here too the yield of T7 per bacterium is much smaller due to some interference from T1.

Similar depressor effects have been found for other pairs of viruses. It appears that the excluded virus can exert its depressing influence even when it is added rather late, when the first virus is well along its course of multiplication.

Another kind of yield effect has been observed in a very special case. I must preface the description of this phenomenon by mentioning a discovery of Dr. A. D. Hershey. Dr. Hershey (personal communication) discovered that the virus strains T2, T4, and T6 can each occur in two modifications, each of which appears to be, under most conditions, hereditarily stable. The two modifications of T2, for instance, may be looked upon as arising one from the other by mutation. The two modifications give plaques of different appearance and differ also in certain other respects. The two modifications are called $T2r^+$ and T2r.

The yield effect found by us (unpublished) is of the following kind. Suppose a bacterium is infected simultaneously with $T2r^+$ and T4r. There will be mutual exclusion at least to a large extent, that is, there will be bacteria liberating T2 and bacteria liberating T4, and few if any bacteria which liberate both. There will also be a depressor effect in that those bacteria which liberate T4 liberate a reduced number of T4 particles, due to interference from T2. These are effects of the type described just previously. However, there appears to be here a third kind of interference effect. The bacteria which liberate T4 liberate *a mixture of* $T4r^+$ *and T4r* although they had been infected with pure T4r. It appears that $T2r^+$, while unable to multiply, can induce in T4r, in the mixedly infected bacteria, the mutation from T4r to $T4r^+$. The study of this phenomenon is still incomplete.

The mutual exclusion effect is of so novel a type that its explanation calls for a bold hypothesis. We assume (4, 16) that the first virus which penetrates the cell wall makes the cell wall impermeable to other virus particles, just as the fertilization of an egg by one spermatozoon makes the egg membrane impermeable to other spermatozoa. I am mentioning this analogy merely as an aid for the visualization of our hypothesis, without in any way wishing to imply a biological significance.

If such a membrane change is accepted as the explanation of the mutual exclusion effect, the various yield effects have to be ascribed to an ability of the excluded virus to interfere with the multiplying virus even though it is separated by the membrane. At first sight this may be difficult to believe. However, the assumption that the excluded virus is located on the outside of the cell membrane is supported by another observation (16): the "depressing" action of the excluded virus can be blocked by specific antiserum, and we know that antiserum does not act on intracellular virus.

Time does not permit to argue the merits of this "penetration hypothesis," and still less to give reasons for the rejection of alternative hypotheses. I will confine myself to summarizing the penetration hypothesis in figure 12.

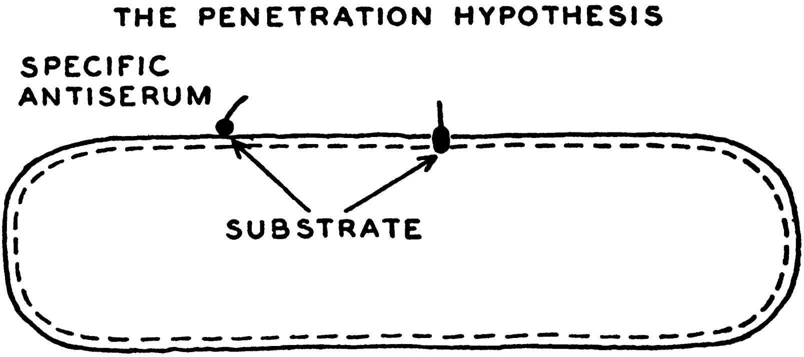

FIG. 12. Schematic representation of the penetration hypothesis. One virus particle has penetrated the cell wall. As a result of this penetration the cell wall has become impermeable to other virus particles. Another virus particle has become adsorbed on the outside of the bacterium. In this position it can interfere with the multiplication of the virus which penetrated into the interior. This interfering action of the second virus can be blocked by the action of specific antivirus serum.

I would like to turn now to a side issue of the research on bacterial viruses, a side issue which is threatening to displace the main issue, by virtue of its explosive content of possibilities for studying bacterial genetics.

We have previously mentioned the use of indicator strains in experiments with mixed infections. These indicator strains are obtained as derivatives of the original "wild-type" bacterial strain *B* with which all our experiments have been conducted. They are obtained as secondary growth, i.e., as growth in cultures in which the wild-type has been lysed away by the action of one or the other of the viruses.

It could be shown (18) that the virus which is added to eliminate the wild-type acts merely as eliminator. It does not *induce* the change from sensitivity to resistance in those bacteria which survive and proliferate.

If it is not the added virus which is the agent inducing the change from sensitivity to resistance what is the agent? Apparently these changes are similar to the spontaneous mutations of higher organisms. The occurrence of the mutations is random, but with fixed probabilities if the cultural conditions are held constant. You may think, if you wish, of such a mutation as a molecular change in a gene, requiring for its initiation a rather high activation energy, or you may think of any other kind of miscarriage of a normal process, reflecting a limitation in the devices employed by the cell to safeguard its normal reproduction.

Mutations from sensitivity to resistance delight the heart of the experimenter because of the marvellous ease with which the mutants can be isolated from cultures in which they occur as rarely as one in one hundred million of the bacteria. A standard procedure is to spread the bacteria together with an excess of virus on nutrient agar. After incubation, the sensitive bacteria are lysed and the resistant bacteria have formed colonies from which the mutants can be isolated. Try to imagine what the chromosome map of the fruitfly would be like if Morgan, Bridges, and Sturtevant had had a means of examining one hundred million flies at a time!

The wild type bacterium may be capable of different mutations all of them involving resistance to one particular virus. If the rates of these different mutations are not too different they may be picked up in the same plating. We will discuss two examples of this type of diversity in the mutation pattern (figure 13).

When resistance to virus T1 was first studied (18) it was noted that the resistant colonies seemed to be classifiable into "large colony formers" and "small colony formers." This, of course, is a poor method of classification as the colony size depends on the time of incubation, medium, etc., but at the time of this first study Dr. Luria and I were unable to find other distinguishing characteristics for these two types. Since that time four such characteristics have been found. First, Demerec and Fano (19) found that the "large colony former" is always resistant not only to T1 but also to T5, while the "small colony former" is not. Following Demerec's and Fano's notation, the two mutations can therefore be designated as *B*/1,5 (the large colony former) (from (1) to (2), figure 13) *B*/1 (the small colony former) (from (1) to (3), figure 13). Second, Luria (20) found that *B*/1 is attacked by a mutant of the virus T1, called T1′, and that *B*/1,5 is not sensitive to this mutant of the virus. Third, E. H. Anderson (21a) found that the mutant *B*/1 requires a growth factor, tryptophane, which the other mutant, *B*/1,5, does not require. Fourth,

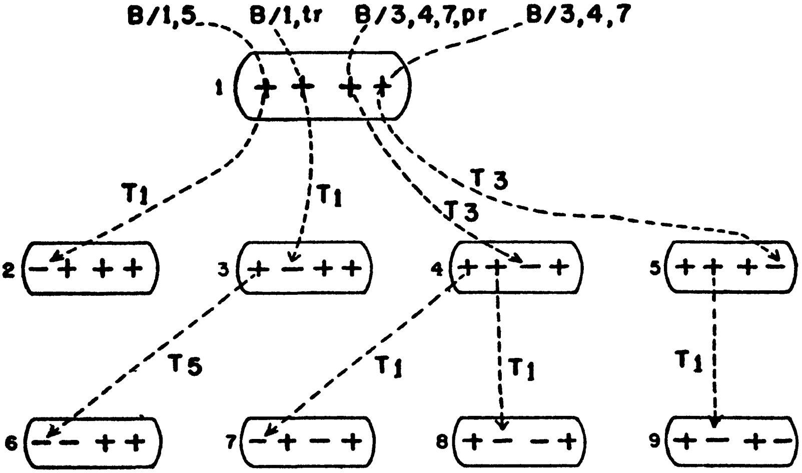

FIG. 13. One-step and two-step mutations of *B*. For explanations see text.

E. H. Anderson also found (21a), that *B*/1, in contrast to *B*/1,5 cannot utilize $NH_4^-$ as a nitrogen source in the absence of donors of amino nitrogen.

The two mutations, from *B* to *B*/1 and from *B* to *B*/1,5, represent, therefore, two entirely distinct alterations in the hereditary make-up of the cell. These two alterations are, furthermore, independent of each other, i.e., a bacterium in which one of these mutations has occurred is still capable of the other mutation, and, in fact, the second mutation will occur with the same rate, irrespective of whether the first mutation has occurred or not (19, 20). Thus, if we plate a large number of bacteria of the mutant strain *B*/1 with the virus T5 we can isolate from the secondary colonies a strain which has a superposition of the characteristics of the two mutations. This two-step mutant will be resistant to T1 and T5, and to T1′, and will be deficient for the growth factor tryptophane (from (3) to (6), figure 13).

We will note in passing that the two viruses T1 and T5 here referred to are quite unrelated viruses. The electron-microscope shows that they differ by about a factor two in their diameters. Specific antisera to these two viruses give no cross reactions whatsoever. At first sight it might appear strange that the bacterium should be capable of a mutation which causes it to become resistant simultaneously to two unrelated viruses. Many a man, in fact, might want to infer from the occurrence of such a mutation that there does exist a recondite relationship between the two viruses which for some reason is not apparent from the electron-microscope pictures or from the serological tests. I believe that such an inference would be unsound, because there is nothing to support it. I believe we should rather look upon the resistances to T1 and T5, respectively, as different characters affected by the same genetic change (21). It is a commonplace in genetics that every genetic change affects many characters even though only one or a few of these changes may be so gross as to force itself on our attention. As an example we may cite the mutant *r p* of Drosophila melanogaster. In animals carrying this mutation the genitalia of the male are rotated counterclockwise through 180° and the eyes are roughish. We may infer from this that there are interrelationships in the processes which lead to the determination of the genitalia and of the eyes, respectively. Similarly, we infer from the occurrence of the mutation from *B* to *B*/1,5 that there are interrelations in the processes which determine sensitivity of the bacterium to T1 and T5, respectively. However, we have no basis for inferring a similarity between the two viruses T1 and T5 themselves.

If we select mutants of *B* which are resistant to the virus T3, we obtain mutants which are not only resistant to T3 but also to T4. Here again viruses T3 and T4 are morphologically distinct and serologically unrelated. In most cases these mutants will also be resistant to T7. These mutants, which should be designated as *B*/3,4,7, are of two types. One type, which requires no growth factor (from (1) to (5), figure 13) and another type which requires proline (McCormick and Doermann, unpublished) (from (1) to (4), figure 13).

From either of these two mutants, those requiring no growth factor and those requiring proline, we can obtain a variety of two-step mutants by selection in the presence of T1. From the one requiring no growth factor, for instance, we obtain a two-step mutant resistant to T3, T4, T7, and T1 which requires tryptophane ((5) to (9), figure 13), and from the one requiring proline, we can obtain one having the same resistance pattern and requiring proline and tryptophane ((4) to (8), figure 13) (E. H. Anderson, unpublished), and also another one, resistant to T3, T4, T7, T1, and T5, and requiring proline only ((4) to (7), figure 13).

' These examples may suffice to explain the principle of superposition for obtaining two-step and multi-step mutants.[5] It will be clear that the variety of mutants obtainable is very large. Besides the four primary mutants here mentioned there are several others, obtainable by isolations in the presence of other viruses. These have not yet been studied in sufficient detail to ascertain whether they can be segregated further into several classes according to their growth factor requirements.

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[5] In a very interesting series of publications, which has only recently become available in this country, Lwoff and Audureau (23–25) describe a similar principle of superposition for a series of mutations of the organism *Moraxella Lwoffi*. The mutations studied by these authors endow the organism with an extension of the range of carbon compounds at the expense of which the organism can multiply. In a preliminary note (26) the authors refer to experimental evidence which tends to show that at least one of these mutations does not involve the acquisition of a new enzyme by the organism, but merely an alteration in the stability of an enzyme also possessed by the wild-type organism.

Demerec and Fano (19), who have studied the resistance patterns in detail, have also recorded the occurrence of mutations which *apparently* combine two of the primary steps in one step. Thus, there are mutations which make the organism in one step resistant to the viruses T1, T5, T3, T4, and T7, thus apparently combining in one step the two steps leading from *B* to *B*/1,5 and from *B* to *B*/3,4,7. These "two-in-one" step mutations occur more rarely than either of the two one-step mutations referred to, but are not so rare by far that one could explain them as random coincidence of the two mutations in one organism. It remains to be seen whether the two-in-one mutations are genuine genetic superpositions of the two individual mutational steps, or whether the superposition is only a phenotypic one, which may be caused by an independent genetic change.

Progress in this field will require careful characterization of the mutants with respect to their resistance patterns and with respect to their growth factor requirements. I will give two examples to illustrate the necessity of detailed characterization.

If one tries to isolate mutants resistant to T2, i.e., mutants of the type *B*/2, one finds that these mutants very quickly lose their resistance to T2. Sometimes the strain is found to be sensitive after one or two successive streaks which have to be made to free the strain from the virus in whose presence it was isolated. Apparently, therefore, the mutants *B*/2 mutate in reverse, back to *B*. Closer study of the resistance pattern shows however that the apparently backmutated strain is resistant to T6 (E. H. Anderson, unpublished). The sequence is probably this: from *B* to *B*/2,6. This mutant is itself unstable, i.e., it has a high mutation rate. It does not, however, revert to *B*, but to another form, resistant to T6 only.

Of the physiological tests which may serve to differentiate mutants we have only mentioned growth factor requirements. Growth factor requirements are indeed convenient and striking differentiators, and their occurrence is by no means rare. A careful search for other physiological differentiators by Miss Ann Martin (22) has been unrewarding with one exception.[6] Miss Martin has tested 13 of Dr. E. H. Anderson's mutants by a large number of the customary fermentation tests employed in the identification of E. coli. Although she has found some minor, mostly quantitative, differences between some of the strains in these tests, there was on the whole a remarkable uniformity of reaction.

The study of changes in virus resistance and in physiological characters should lead on to interesting population and evolution studies of bacteria. At present, however, the main task is still the closer study of the mutations.

I hope I have made it clear in this presentation of some experiments with bacterial viruses that the field is wide open for further studies. The biochemical study of virus growth, although obviously within the reach of present techniques, has hardly begun. The study of interference has led to startling discoveries, whose interpretation is yet uncertain. Here the techniques of study are farthest developed. In bacterial genetics the ground has been broken by the proof that changes from virus sensitivity to virus resistance are due to spontaneous mutations, and by showing that the mutants exhibit correlated physiological changes. The task of exploiting this spade work is still ahead, and seems full of promise. A strong feeling of adventure is animating those who are working on bacterial viruses, a feeling that they have a small part in the great drive towards a fundamental problem in biology.

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[6] The differences in the cultures grown in milk provide a definite basis for a division of the strains which Miss Martin studied into three groups: (1) Those that react as did the parent strain, with a maximal production of acid, gas, and hard curd, (2) those which formed a soft curd, less acid, and no gas (except in one instance), and (3) those which produced neither curd nor gas. It was found that all the mutants in the second group are resistant to T7, although not all mutants resistant to T7 follow this pattern. Further, all the organisms in the third group are resistant to T1, and that this group includes all the strains in which the second locus of figure 13 (the locus whose mutation causes resistance to T1 and the tryptophane growth factor requirement) had mutated.

## LITERATURE CITED

1. Delbrück, M., *Biological Reviews,* 1946, **21**, 30.
2. Delbrück, M., *J. Bact.*, 1945, **50**, 137.
3. Gratia, A., *Ann. Inst. Pasteur,* 1936, **57**, 652.
4. Luria, S. E., and Anderson, T. F., *Proc. Nat. Acad. Sc.*, 1942, **28**, 127.
4a. Luria, S. E., Delbrück, M., and Anderson, T. F., *J. Bact.*, 1943, **46**, 57.
5. Delbrück, M., and Luria, S. E., *Arch. of Biochem.*, 1942, **1**, 111.
6. Ellis, E., and Delbrück, M., *J. Gen. Physiol.*, 1939, **22**, 365.
7. Burnet, F. M., *Brit. J. Exp. Path.*, 1929, **10**, 109.
8. Delbrück, M., *J. Bact.*, 1945, **50**, 131.
9. Henle, W., and Henle, G., *Science,* 1943, **98**, 87.
10. Henle, W., and Henle, G., *Amer. J. Med. Sci.*, 1944, **207**, 705.
11. Henle, W., and Henle, G., *Amer. J. Med. Sci.*, 1944, **207**, 717.
12. Ziegler, J. E., and Horsfall, F. L., *J. Exp. Med.*, 1944, **79**, 361.
13. Ziegler, J. E., Lavin, G. I., and Horsfall, F. L., *J. Exp. Med.*, 1944, **79**, 379.
14. Schlesinger, R. W., Olitsky, P. K., and Morgan, I. M., *J. Exp. Med.*, 1944, **80**, 197.
15. McKinney, H. H., *Amer. J. Bot.*, 1941, **28**, 770.
16. Delbrück, M., *J. Bact.*, 1945, **50**, 151.
17. Luria, S. E., and Delbrück, M., *Arch. of Biochem.*, 1942, **1**, 207.
18. Luria, S. E., and Delbrück, M., *Genetics,* 1943, **28**, 491.
19. Demerec, M., and Fano, U., *Genetics,* 1945, **30**, 119.
20. Luria, S. E., *Genetics,* 1945, **30**, 84.
21. Anderson, E. H., *Proc. Nat. Acad. Sci.*, 1944, **30**, 397.
21a. Anderson, E. H., *Proc. Nat. Acad. Sci.*, 1946, **32**, 120.
22. Martin, Ann, *Vanderbilt Univ. Thesis for the Degree of M.A.*, 1946.
23. Lwoff, A., and Audureau, A., *Ann. Inst. Pasteur,* 1941, **66**, 417.
24. Lwoff, A., and Audureau, A., *Ann. Inst. Pasteur,* 1941, **67**, 94.
25. Audureau, A., *Ann. Inst. Pasteur,* 1942, **68**, 528.
26. Lwoff, A., and Audureau, A., *Ann. Inst. Pasteur,* 1944, **70**, 51.

# RADIOACTIVITY AND NAGASAKI[1]

STAFFORD L. WARREN

*Colonel, U. S. Army; Professor of Radiology, University of Rochester*

COLONEL WARREN, because of his army duties and obligations as medical officer of the Manhattan Project, was unable to prepare a manuscript for presentation and publication.

He showed the army's official motion picture records of the results of the atomic bomb at Nagasaki, with a running comment, and photographs of some of the material damage and human victims. These pictures were extraordinarily interesting and informative, but are not available for reproduction in our record. The photographs gave clear indication of pigmentation from infra-red radiation, epilation from gamma radiation, and petechiae. Many of the cases showed marked cytopenia and extensive destruction of bone marrow.

Colonel Warren discussed briefly the medical services given to the communities at Oak Ridge, Hanford and Los Alamos, the protection of personnel against the plant hazards in the production of the atomic bomb, the teamwork required for research in the biologic effects of the radiations, and the effectiveness of the protective methods used. He gave a vivid description of his experiences in the Alamogordo Desert when the first bomb was exploded. After the Japanese surrender Col. Warren went to Hiroshima and Nagasaki with his colleagues, landing ahead of the troops, to inspect the damage from the bombs and assay the hazards for the troops.

He closed his lecture with a stirring plea that we "make the United Nations Organization work," in order that it may never again be necessary to use the atomic bomb in warfare.

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[1] Lecture delivered February 21, 1946.

# BIOLOGICAL ANTAGONISMS BETWEEN METABOLICALLY IMPORTANT COMPOUNDS AND THEIR STRUCTURAL ANALOGS[1]

D. W. WOOLLEY

*Associate, Rockefeller Institute for Medical Research*

DURING the past few years compounds which are very similar in structure to vitamins, hormones, and other metabolically important substances, have been synthesized or found to exist in nature. These structural analogs have the property of calling forth in various living organisms some or all of the signs associated with deficiency of the metabolite to which they are related. Tonight I wish to discuss with you some of the facts in this regard which we have chanced upon, and to trace very briefly the origins of the concepts. I would also like to explore with you some of the theoretical and practical aspects in biology arising from this work.

My own interest in the antagonism between structurally related compounds goes back to the winter of 1937 and 1938. At that time, following our identification of nicotinamide as the pellagra-preventative factor (1), a number of chemicals related to nicotinic acid were assayed for vitamin-potency in order to gain some basis for an opinion of the relationship of structure to vitamin-activity (2). Surprisingly enough, it was found that 3-acetylpyridine was quite poisonous to nicotinic acid-deficient dogs, but was harmless to normal animals. At that time no explanation of these results could be made. At the present time, as we shall see in that which follows, 3-acetylpyridine is believed to cause nicotinic acid deficiency by interfering with the action of the vitamin. In looking back over the literature one can see that other investigators likewise had made a few scattered notes on the antagonism which certain structurally similar compounds exerted against certain biologically important substances. Some of these findings seemed to run so counter to modes of thought then in vogue that doubts were cast on the validity of the observations. Such was the case as late as 1940 with regard to Kuhn's claim that the sex-determining factor in certain algae was not a single agent but rather the ratio between cis and trans dimethyl crocetin (3). Today, the dependence of a specific biological response on the *ratio* between two closely allied compounds does not seem so heretical.

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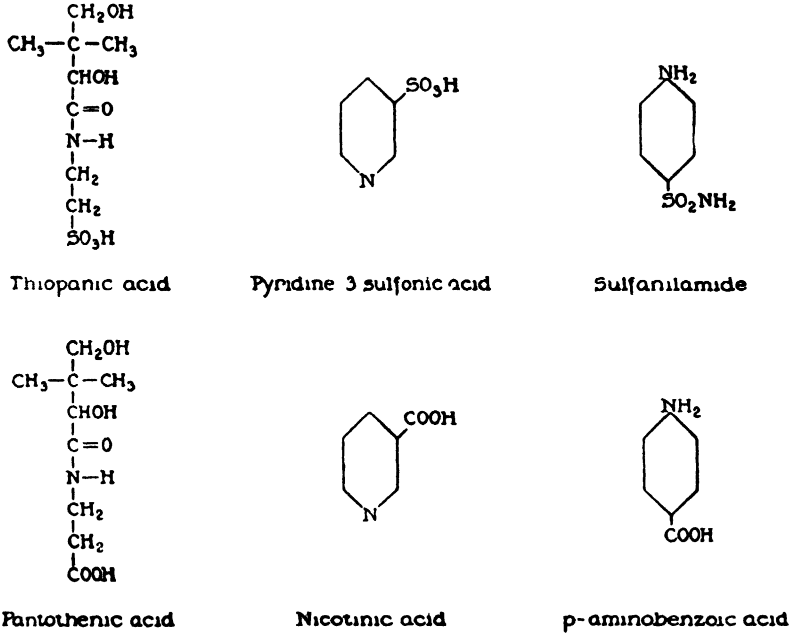
[1] **Lecture delivered March 21, 1946.**

In 1937 Clark (4) very clearly set forth his idea that antagonism which could be demonstrated between certain structurally similar pharmacological agents was due to competition between them for a specific part of the cell. Clark's views did not become widely known until very recently.

Attention was centered on the competition between structural analogs by Woods' discovery that the bacteriostatic action of the sulfonamides could be negated by additions of p-aminobenzoic acid (5). This latter compound was quickly shown to be a normal constituent of cells, and an essential growth-factor for many microorganisms. It was postulated that sulfanilamide owed its bacteriostatic action to the production of a crippling deficiency of the essential metabolite, p-aminobenzoic acid. This action was possible because of the analogous chemical structures of the two substances.

It was the involvement of a dramatic new therapeutic agent namely, sulfanilamide, in the slowly forming concepts of competition between metabolites and compounds related to them structurally, that focused attention on this biological phenomenon. Without this impetus, progress would have been much slower.

Following Woods' discovery, Fildes (6) showed that alteration of the tryptophane-molecule to give indole acrylic acid would yield an antibacterial agent. McIlwain demonstrated that the same structural change involved in passing from the metabolite, p-aminobenzoic acid to sulfanilamide would, when applied to nicotinic acid, give rise to a bacteriostatic agent, pyridine-3-sulfonic acid, the action of which was antagonized competitively by nicotinic acid (7). Snell observed the same situation with pantothenic acid and thiopanic acid (8). The structural relationships involved in these examples may be seen in the first slide (figure 1).



Thiopanic acid Pyridine 3 sulfonic acid Sulfanilamide

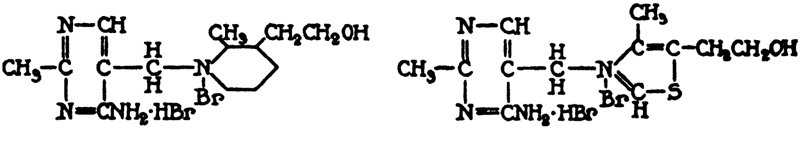
Pantothenic acid Nicotinic acid p-aminobenzoic acid

FIG. 1. Structural formulae of some metabolites and related analogs.

We also had been concerned with thiopanic acid in relation to pantothenic acid (9), and had done experiments on the possible usefulness of this agent in the treatment of virus diseases. Therefore, it was natural to look for some more compounds that would compete with vitamins or other metabolites. Let us now examine some of the things that were found.

A series of compounds was soon discovered which would call forth in animals the signs characteristically associated with specific vitamin deficiencies. Let us discuss in some detail one of the first of these, for many of the features of antagonism between structurally related compounds will thus be illustrated.

When the sulfur atom of thiamine is replaced with a vinyl group, the pyridine analog of the vitamin, pyrithiamine (10), is obtained. The relationship in structure between these two substances is shown in the next slide (figure 2). Following a suggestive experiment of Robbins with a fungus (11), we prepared this compound and fed it to mice. A few days after the administration was begun, the animals became unable to stand upright on their hind legs, and would topple over backward when they attempted to do so. They soon became hyperirritable, their appetites dwindled, and presently they would be thrown into convulsions either spontaneously, or especially when picked up by the tail. Opisthotonos, familiarly seen in thiamine deficiency, became evident and eventually extensive prostration ensued. In the terminal stages of the disease, the mice assumed a characteristic position in which their legs were stretched out on either side at right angles to the body, and only the head was responsive to stimuli. Death soon followed. These signs have been seen frequently in various species as the characteristic manifestations of thiamine deficiency. When increased amounts of this vitamin were added to the basal diet, the disease called forth by pyrithiamine administration was averted. Likewise, thiamine was effective in curing animals, and would even resuscitate them dramatically from the terminal stages. Data to illustrate the competition between pyrithiamine and thiamine in mice are shown in the next slide (table 1).



Pyrithiamine Thiamine

FIG. 2. Structures of pyrithiamine and thiamine.

Pyrithiamine also proved to be a rather active agent for the suppression of growth of many microorganisms. Here too its action was prevented by increasing the thiamine-content of the medium (12).

From a quantitative standpoint, the relationship between this pair of compounds is interesting, for it can be seen that the action of pyrithiamine did not depend on the absolute amount present, but, for any given species, it was decided by the ratio of pyrithiamine to thiamine. In other words, pyrithiamine competed with thiamine for the attention of the organism. Such a competitive situation is almost universal between metabolites and their inhibitory structural analogs.

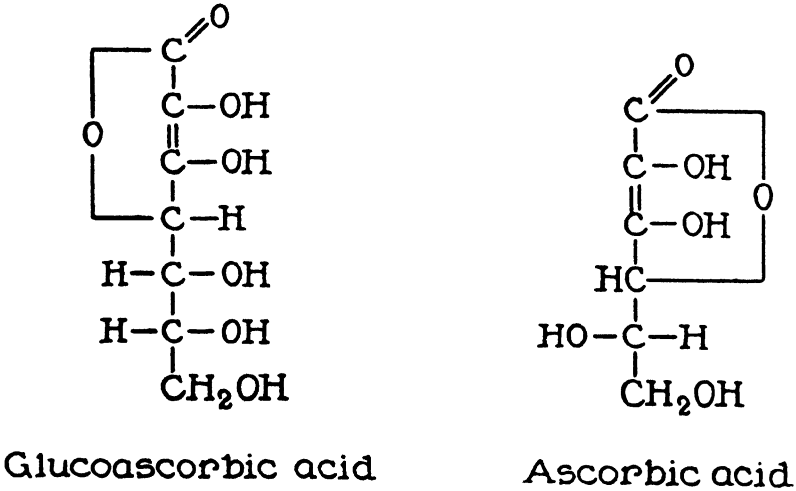
TABLE 1

*Response of Mice to Various Doses of Thiamine and Pyrithiamine*

| Pyrithiamine | Thiamine | Animals showing deficiency signs | Average change in weight |
|---|---|---|---|
| *gamma per day* | *gamma per day* | *per cent* | *gm. per wk.* |
| 0 | 1.6 | 0 | + 3.0 |
| 600 | 1.6 | 100 | − 0.2 |
| 300 | 1.6 | 100 | + 1.9 |
| 100 | 1.6 | 100 | + 2.5 |
| 100 | 2.0 | 75 | + 2.4 |
| 50 | 2.0 | 0 | + 3.5 |
| 600 | 61.6 | 0 | + 3.1 |
| 2000 | 60.0 | 0 | + 3.6 |

Although it may seem irrelevant, I cannot refrain from a few words about the importance to these studies of a knowledge of nutrition, and more especially, the development of highly purified adequate diets for animals, and chemically defined media for microorganisms. Most, if not all of the phenomena we are to discuss tonight would not have been found had it not been for the tedious and difficult work which has been done to ferret out, to isolate, and to characterize the essential nutritive requirements of animals and microorganisms. It was necessary to know and to manipulate the thiamine-content of the ration in order to carry out the experiments with pyrithiamine. In like manner, synthetic diets and media have been essential to the elucidation of the other facts to be discussed tonight.

After the demonstrations with pyrithiamine, analogs of other vitamins were produced which were found to cause manifestations of disease similar to those seen in deficiency of the related vitamin. These diseases were believed actually to be deficiencies not only because of the similarities in pathology, but also because they were prevented or cured by adequate amounts of the vitamins concerned. Hand in hand with these experiments on animals a number of studies were made with microorganisms as the biological means of detecting and exploring the phenomenon of antagonism. Let us briefly mention some of these cases.


Glucoascorbic acid

Ascorbic acid

FIG. 3. Structures of glucoascorbic acid and ascorbic acid.

Glucoascorbic acid, the structure and relationship of which to ascorbic acid are shown in the next slide (figure 3), was found to cause a condition in mice with many of the signs of scurvy as seen in animals susceptible to that disease (13). Now, the mouse, like most other animals, does not need ascorbic acid in the diet. Therefore, a scorbutic mouse has never been recognized, but the individual shown in the next slide (figure 4) had most of the types of lesions seen in scorbutic guinea pigs or primates. Guinea pigs were also susceptible to the action of glucoascorbic acid, and in this species ascorbic acid counteracted its effect (14).

Shortly thereafter benzimidazole was investigated because the pharmacological action of it on animals had just been reported by Goodman and his associates (15). This drug caused extensive loss of muscular tone, sufficient to immobilize the animal. It was therefore of interest to find that the benzimidazole was an inhibitor of microbial growth, and that adenine or guanine antagonized this action (16). Data to illustrate these points are shown in the next slide (table 2). The structural relationship of the drug to adenine may be seen in the next slide (figure 5). The reason I referred to this case as interesting will appear when it is recalled that adenine in the form of adenosine tri-

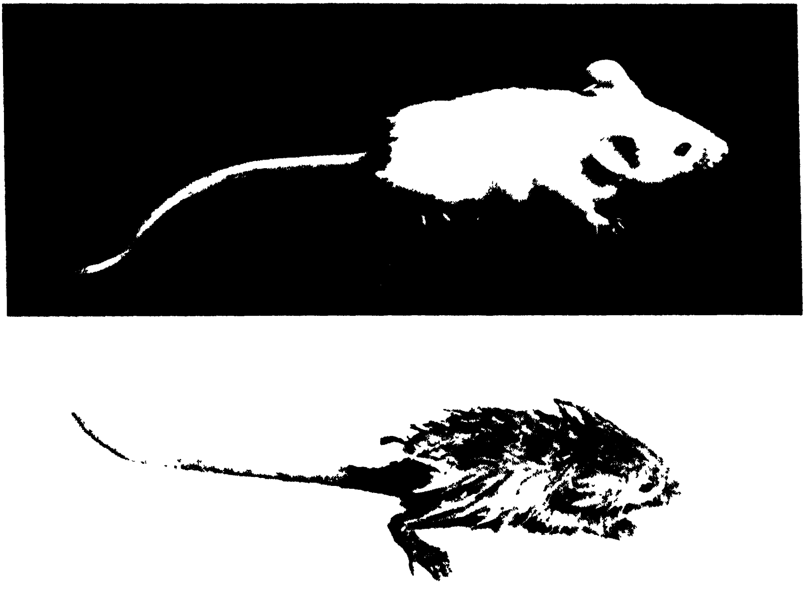


FIG. 4. **Mouse treated with glucoascorbic acid compared to control.**

TABLE 2

*Inhibitory Effects of Graded Amounts of Benzimidazole on Growth of Saccharomyces cerevisiae and Their Reversal by Adenine*

| Benzimidazole | Adenine sulfate | Turbidimeter reading* |
|---|---|---|
| *γ per cc.* | *γ per cc.* | |
| 0 | 0 | 69 |
| 100 | 0 | 69 |
| 200 | 0 | 66 |
| 300 | 0 | 84 |
| 500 | 0 | 96 |
| 600 | 0 | 99 |
| 1000 | 0 | 100 |
| 600 | 1000 | 69 |
| 600 | 600 | 83 |
| 600 | 300 | 93 |
| 1000 | 2000 | 80 |

* Turbidimeter readings were expressed as per cent of the incident light transmitted by the cultures, and hence were inversely proportional to the amount of growth.

phosphate and adenylic acid plays a prominent role in muscular contraction, and muscular contraction is inhibited by benzimidazole. Nevertheless, adenine will not overcome the action of benzimidazole in animals, but will do so only in microorganisms.

To continue with examples of antagonism, signs of riboflavin-deficiency were produced in mice as well as in various bacteria

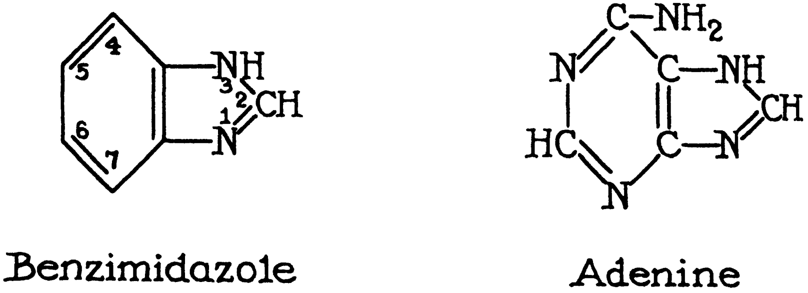
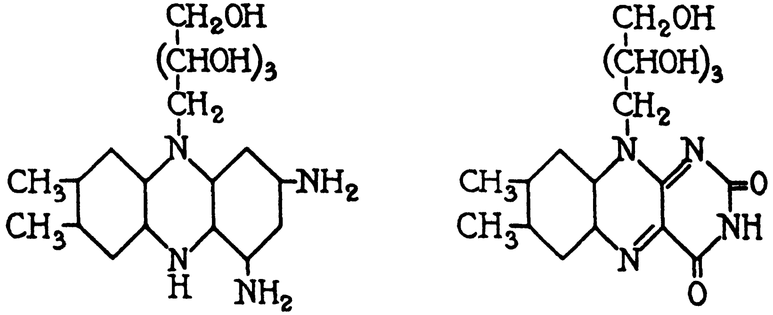


FIG. 5. Structures of benzimidazole and adenine.

by the administration of the phenazine analog of the vitamin shown in the next slide (figure 6) (17). This compound was prepared and tested because of the previous results with benzimidazole. Since the exchange of a pyrimidine ring in adenine for a benzene ring to form benzimidazole had produced a successful antagonist, the same type of structural alteration was applied to riboflavin, and the result was the same. It was the pyrimidine ring contained in the right hand side of the riboflavin structure that was replaced by the benzene ring in order to form the phenazine.



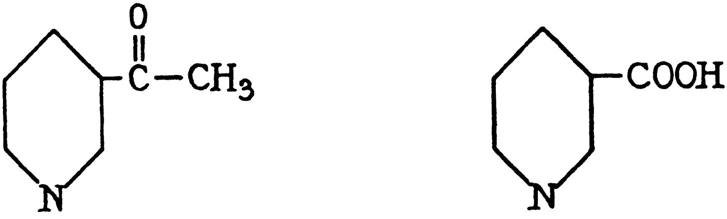
2,4-diamino-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine

Riboflavin

FIG. 6. Structures of riboflavin and its phenazine analog.

During all this time the experiments with acetylpyridine in nicotinic acid-deficient dogs had not been forgotten. These were the ones outlined at the beginning of this lecture. The matter was taken up again, and it was soon demonstrated that this compound did act in competition with the vitamin (18). Since mice do not require dietary nicotinic acid, no one knows how the deficiency should manifest itself in this species Nevertheless, the feeding of 3-acetylpyridine called forth such signs in mice as fiery red tongues, diarrhea, dermatitis, loss of weight and death. Such findings are characteristic of pellagra and black tongue. These manifestations were prevented by the inclusion of more nicotinic acid in the ration. The next slide (figure 7) will show the structural similarity of the two substances.

Now, if one applied to pantothenic acid the same type of structural change as was involved in passing from nicotinic acid to 3-acetylpyridine, the formation of an inhibitory structural analog of pantothenic acid might be expected. This is particularly so when it is known that Auhagen (19) observed that this alteration converted p-aminobenzoic acid into p-aminoacetophenone, an antagonist of that metabolite. However, when the desired analog of pantothenic acid was synthesized, it did not behave as an inhibitor. More careful examination of the cases of 3-acetylpyridine and of p-aminoacetophenone indicated that while these were aromatic type ketones and hence somewhat more acidic than aliphatic ketones, the pantothenic acid analog was a purely aliphatic substance. Therefore, an aromatic type of analog was formed by synthesizing phenyl pantothenone, and this proved to be a valuable competitor to pantothenic acid (20). Its value will appear later in the discussion. The structure of phenyl pantothenone and its relationship to that of pantothenic acid are shown in the next slide (figure 8).



3-acetyl pyridine

Nicotinic acid

FIG. 7. Structures of 3-acetylpyridine and nicotinic acid.

I have bedecked this slide purposefully with many formulae, not only to show you the three separate ways in which the analog was prepared, but also to call attention to a phase of the work which is not being discussed tonight. A few of the compounds which one would wish to test as metabolite antagonists can be purchased from supply houses. A few more can be synthesized according to directions in the chemical literature. By far the majority, however, have never been described, and it is therefore necessary to conduct investigations in pure organic chemistry before the biological work is even attempted. For this reason, a major part of the effort involved has been in synthetic organic chemistry. Since the title of my paper tonight begins with "biological," little can be said of the purely chemical aspects.

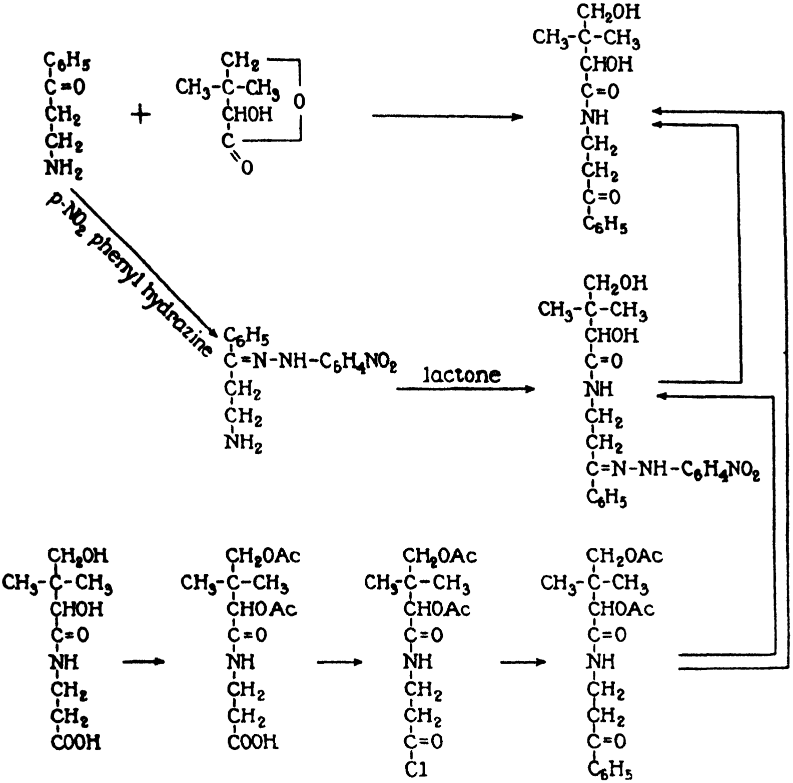
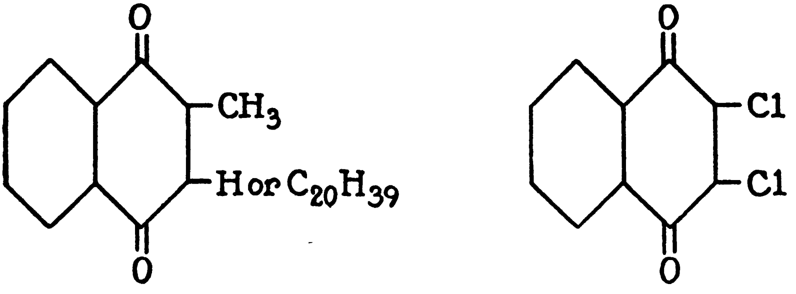


FIG. 8. Structure and modes of synthesis of phenyl pantothenone.

As a final example of an antagonist to a vitamin let us consider the case of 2,3-dichloro-naphthoquinone. During the war, the U. S. Rubber Company introduced this compound as an antifungal agent (21). It was developed empirically just as were the sulfonamide drugs. When we heard of this new and useful fungicide, the similarity of its structure to that of vitamin K seemed so striking that experiments were begun to determine whether its action could be antagonized by the vitamin. The next slide (figure 9) will show the structures of the two substances. The dichloro-naphthoquinone was the most powerful chemical ever examined for inhibition of the growth of yeasts (22). As expected its growth-inhibiting action on yeast was reversed competitively by vitamin K. These points are illustrated by the data in the next slide (table 3).



Vitamin K

2,3-dichloro-naphthoquinone

FIG. 9. Structures of 2,3-dichloro-naphthoquinone and vitamin K.

Let us turn now from a brief scanning of examples of antagonism between metabolites and their analogs to a consideration of some general features of this biological phenomenon. I shall attempt to state briefly some of the general principles that have appeared from a mass of data which has been collected by various workers over the past few years. Few of these generalizations will be found free of exceptions. Indeed, our knowledge of this field is so fragmentary that in a few years the picture may look quite different from the one before us tonight. Therefore, I shall endeavor to reduce hypotheses to a minimum. The task of outlining the general principles of antagonism between structurally related compounds is similar to that of the biochemist who attempts to summarize the salient points concerning enzyme-action. In both instances there is no single criterion which can be set up to define the phenomena, because exceptions to almost every rule can be found. About all which can be said is that there are a number of features usually associated with biological antagonism between structural analogs, just as there are with enzyme action; and that a majority of these characteristics apply to any given specific case.

TABLE 3

*Effect of 2,3-dichloro-naphthoquinone and of 2-methyl-naphthoquinone singly and together on the growth of Saccharomyces cerevisiae*

| 2,3-dichloro-naphthoquinone | 2-methyl-naphthoquinone | Turbidity* of culture |
|---|---|---|
| *μg. per cc.* | *μg. per cc.* | |
| 0.0 | 0.0 | 39 |
| 0.002 | 0.0 | 78 |
| 0.005 | 0.0 | 93 |
| 0.01 | 0.0 | 99 |
| 0.005 | 0.04 | 60 |
| 0.005 | 0.02 | 68 |
| 0.005 | 0.01 | 77 |
| 0.005 | 0.005 | 85 |
| 0.002 | 0.02 | 48 |
| 0.002 | 0.01 | 66 |
| 0.0 | 0.05 | 46 |
| 0.0 | 0.20 | 65 |

* Turbidity is expressed as per cent of the incident light transmitted by the culture when the uninoculated basal medium is considered to have 100 per cent transmission.

In the first place, antagonism between a metabolite (such as a vitamin or hormone) and its structural analog is usually competitive. This is best stated by saying that the biological action of the agent is dependent, not on the absolute amount present, but rather on the ratio of the quantity of the analog to that of the metabolite. This ratio is constant, at least over a limited range of concentration, and when this is so the antagonism is said to be competitive. The ratio between the concentration of analog necessary to cause a biological response and that of the metabolite needed to reverse or negate it exactly is called the inhibition index. It follows that for a series of inhibitory analogs of a given metabolite, the smaller the inhibition index, the more active is the inhibitor. The index is a function of the particular biological system, and may vary widely from species to species for a given inhibitor-metabolite pair. With but few exceptions the index is greater than 1. In other words, much more of an inhibitory analog must be added to an organism than there is of metabolite present. Failure to appreciate this point has led to several faulty conclusions. For example, it would be hazardous to state that an analog of, let us say, vitamin K was not an inhibitory agent, if only 10 or 100 moles of the analog had been supplied for every mole of vitamin in the organism since inhibition indices greater than 10,000 are not uncommon.

From a consideration of these facts one can see that the potency of any given inhibitor depends on two things: first, the inhibition index, and second, the potency of the metabolite concerned. If the metabolite is very active, such as is biotin, and thus only a minute amount suffices to the organism, the chances of producing a very active inhibitory structural analog are greater than if the metabolite is a relatively inactive one such as ascorbic acid. One reason why the sulfonamides are so effective is because of the high potency and consequent low cellular concentration of p-aminobenzoic acid.

Competition between structural analogs cannot be taken as the sole criterion of the phenomenon we are discussing (i.e., of antagonisms). If the action of an agent is reversed by its structurally related metabolite, there is no question; but if reversal is not possible one cannot conclude that the effect of the analog is divorced from that of the metabolite. For example, analogs such as phenyl pentothenone and glucoascorbic acid behave competitively with the related vitamins in some species but not in others. Even in the classical case of sulfonamides and p-aminobenzoic acid there are bacteria known for which reversal of the action of the drugs cannot be effected (23). What shall we conclude when we have tested an analog of some metabolite and have found that it is indeed inhibitory to bacterial growth or otherwise pharmacologically active, but is not counteracted by the related metabolite? If all that we can observe is an inhibition of microbial growth, it does not seem to me that we can conclude with certainty anything about the mode of action. However, if the analog calls forth in animals more or less specific manifestations which have been associated with a deficiency of the related vitamin or hormone, there is some reason to view the agent as interfering with the metabolite even though no reversal can be demonstrated.

A second general feature of antagonism between metabolites and their analogs is the fact that in many instances the only organisms which are affected by the analog are those for which the metabolite is a nutritive essential. If the animal or bacterium can synthesize the metabolite, the structurally similar agent is ineffectual. This dependence of action on nutritive requirement may be seen in the case of pyrithiamine acting on a variety of microorganisms shown in the next slide (table 4) (12). Here it can be seen that the forms which require the vitamin are very susceptible to the growth-inhibition by the analog. Those which are a little less exacting nutritionally, and can get along nicely on the pyrimidine portion of the vitamin-molecule are about ten times more resistant to the action of pyrithiamine. Those which need only the thiazole moiety of thiamine, or the pyrimidine and thiazole parts are even more resistant to the agent. Finally the species which have no requirement nutritionally for the vitamin are unaffected by the analog, and many of these can grow in concentrations of the drug several million times the inhibitory dose for the exacting organisms.

There are several exceptions to this generalization. Inhibitory analogs such as the sulfonamides, benzimidazole, and 2,3-dichloronaphthoquinone do not depend for their action on the nutritional requirements of the organisms. A middle course between these two extremes seems to be followed by analogs such as phenyl pantothenone and glucoascorbic acid, for these act upon all species tested regardless of nutritional needs, but are reversed or competed with by the related vitamin only in the case of those species which require the growth factor. An elucidation of the

TABLE 4

*Inhibitory Power of Pyrithiamine for Various Microbial Species*

| Organism | Inhibition index $\frac{\text{Pyrithiamine}}{\text{Thiamine}}$ | Thiamine requirement |
|---|---|---|
| *Ceratostomella fimbriata* | 7 | Intact thiamine |
| *Ceratostomella* from London plane tree | 19 | " " |
| *Ceratostomella pennicillata* | 10 | " " |
| *Phytophthora cinnamomi* | 12 | " " |
| *Chaloropsis thielavoides* | 11 | " " |
| *Endomyces vernalis* | 130 | Pyrimidine |
| *Mucor ramannianus* | 800 | Thiazole |
| *Saccharomyces cerevisiae* | 800 | Pyrimidine and thiazole |
| *Staphylococcus aureus* | 2000 | " " |
| *Salmonella gallinarum* | 1000 | " " |
| *Neurospora crassa* | Greater than 400,000 | None |
| *Escherichia coli* | " " 2,000,000 | " |
| *Clostridium butylicum* | " " 2,000,000 | " |
| *Lactobacillus arabinosus* | " " 40,000 | " |
| *Lactobacillus casei* | " " 5,000,000 | " |
| *Lactobacillus delbruckii* | " " 5,000,000 | " |
| *Lactobacillus mesenteroides* | " " 5,000,000 | " |
| *Lactobacillus pentoaceticus* | " " 5,000,000 | " |
| *Streptococcus lactis* R | " " 5,000,000 | " |
| *Propionibacterium pentosaceum* | " " 5,000,000 | " |
| *Hemolytic streptococcus* H69D | " " 4,000,000 | " |

underlying mechanisms of these phenomena would be valuable to biochemical understanding.

In order to avoid having this lecture drift off into generalities, I shall discuss only one more common feature of biological antagonism between analogous compounds. Now, although much has been said about the competition between metabolites and compounds similar to them in structure, many of these same analogs are antagonized by naturally occurring compounds totally unrelated to them. The finding of Harris and Kohn (24) that methionine would reverse the microbiostasis caused by the sulfonamides was the first example of this. We have observed that the pharmacological effects of glucoascorbic acid can be counteracted by a nitrogenous substance found in certain fresh plant tissues (13) and that the pellagragenic action of 3-acetylpyridine is as readily negated by tryptophane as it is by nicotinic acid (25). Data to illustrate this are shown in the next slide (table 5). Furthermore, the antimicrobial action of phenyl pantothenone may be reversed by the amino acids histidine, glutamic acid, or proline as well as by pantothenic acid. There may be some significance in the fact that these structurally unrelated antagonists are so frequently specific amino acids. Furthermore, it is noteworthy that these dissimilar antagonists are effective in the cases where the related metabolite fails to counteract the agent. For example, histidine, or glutamic acid, or proline will relieve

TABLE 5

*Responses of Mice to Added Tryptophane and 3-Acetylpyridine*

| dl-Tryptophane added | 3-Acetylpyridine | Survivals | Average change in weight |
|---|---|---|---|
| *per cent of ration* | *mg. per day* | *per cent* | *gm. per wk.* |
| 0.0 | 0 | 100 | +4.0 |
| 0.0 | 4 | 8 | |
| 2.0 | 4 | 100 | +5.2 |
| 0.3 | 4 | 91 | +6.1 |
| 0.1 | 4 | 83 | +4.6 |

the inhibition of growth caused by phenyl pantothenone in either *Saccharomyces cerevisiae* or *Lactobacillus casei,* although, as was discussed above, pantothenic acid is only effective for the latter (or pantothenic acid–requiring) species (26). In like manner, methionine will reverse those bacteriostases by sulfonamides which are not antagonized by p-aminobenzoic acid.

We all wonder about the mechanism of antagonism between structurally related compounds. How does it work? Probably all of you know of the hypothesis advanced by Quastel and Wooldridge (27) and by Clark (4), and made popular by Woods (5) and Fildes (28), which states that the analog competes with the metabolite for a specific site in an enzyme. Either compound can occupy this site at the expense of the other since both, because of structural features in common, can react with the groups involved. When the metabolite combines, it passes normally through the metabolic reactions for which the system is adapted, but when the antagonist unites, it cannot do this. Like the fabled dog in the manger the analog denies the biological system the use of the metabolite. Whether the metabolite or the analog combines with the enzyme depends on relative concentrations in accordance with the law of mass action.

It is not my intention to defend this hypothesis. A few experimental observations have been made which are very difficult to explain in terms of it. Furthermore, in nearly every instance, the postulated enzymes are not known, and so the hypothesis is difficult to examine experimentally. A few models have been set up to demonstrate that the structural analog can actually displace the metabolite from combination with specific proteins. The competition between oxygen and carbon monoxide for hemoglobin, and the recent observation of Dittmer and du Vigneaud (29) that the antagonist biotin sulfone displaces biotin from its combination with antibiotin, may be cited. If these findings can be extended they will add greatly to the prestige of the hypothesis. Since the postulate has been most stimulating to research, I feel that we should adopt it as a working hypothesis until a better one is forthcoming.

Let us turn now to a consideration of the uses to which the knowledge of antagonism between metabolites and their analogs may be put in the understanding of natural processes and in the treatment of disease. Here, although hope exceeds realization, some typical examples of what has already been done will serve to indicate current trends and point the way ahead.

Because the phenomenon of antagonism was popularized by the discovery that the sulfonamides acted in competition with p-aminobenzoic acid, the belief has arisen (28) that other chemotherapeutic agents may be developed against infectious diseases by producing analogs of metabolites. We have already noted that another useful antimicrobial agent, namely 2,3-dichloronaphthoquinone, was found to act in competition with vitamin K, but this discovery came only after the drug had been developed quite empirically. McIlwain and Hawking (30) showed that very large doses of thiopanic acid, an analog of pantothenic acid, were effective in protecting rats against induced streptococcal infection. While this demonstration indicated that there was a possibility of finding a therapeutic agent among metabolite-analogs, thiopanic acid was much too weakly active to be of practical importance.

Now, we had been conducting experiments along this line for several years, and in particular were attempting to develop an inhibitory analog which would be useful in the prevention of infection by obligate, intracellular parasites. For such parasites, in contrast to the situation with pathogenic bacteria, there are, in the main, no therapeutic means of control. For streptococcal or pneumococcal infections the sulfonamides or penicillin seem to be doing a good job; but for poliomyelitis or influenza there are no effective chemotherapeutic agents. I cannot tell you tonight of a drug which is efficacious in these infectious diseases, but there is one which works against an obligate intracellular parasite.

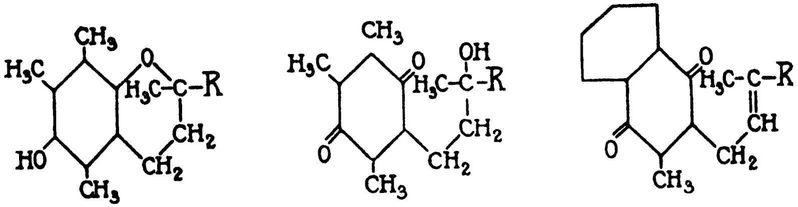
In collaboration with the Survey of Antimalarial Drugs it has been found that phenyl pantothenone, which you will recall is an inhibitory structural analog of pantothenic acid, is about as active as quinine in the treatment of malaria either avian or human. Some derivatives of phenyl pantothenone have been produced which are more active than quinine. Time and opportunity will not permit the telling of this story, except to say that the trial of phenyl pantothenone was prompted by observations on the functioning of pantothenic acid in the metabolism of the parasite. It is only with the conclusion of the war that these matters may be mentioned.

Chemotherapeutic agents are not limited to drugs which are effective against infectious diseases. Indeed, these anti-infection agents form only a small part of the chemotherapeutic arsenal. The application of our knowledge about deficiency diseases, and about their production by inhibitory structural analogs of metabolites, can lead to the formation of new series of pharmacological agents. For example, the specific manifestations which result from a given deficiency of a hormone or vitamin are becoming well known. Some of these signs may have therapeutic desirability. If this is so, it might be possible to produce an antagonistic analog which, as we have seen would bring about the desired sign of deficiency.

But you may ask why the production of signs of deficiency can ever be desirable. Isn't everyone, with vitamin pills and hormone injections, trying to prevent signs of deficiency? To answer this, let us look backward at the case of 3,3′-methylenebis-(4-hydroxycoumarin), the so-called dicoumarol. This agent was observed to produce the specific signs seen in vitamin K deficiency (31). Only after this was established, and it was shown that vitamin K would antagonize the hemorrhagic action of the drug, was it realized that dicoumarol was an analog of vitamin K. The clinical application of dicoumarol is based on the fact that it produces in animals the signs of vitamin K deficiency. With this backward glance to fortify us, let us look ahead to discover whether new series of pharmacological agents may be found among inhibitory structural analogs of metabolites. The history of pharmacology shows that the finding of new types of drugs depends primarily on chance, and that once the first member of the series is thus uncovered, other and possibly more useful derivatives may be synthesized. Perhaps we have a means of coming to the first member of a series without waiting for chance.

As you will recognize, this is nothing but a bright hope. Nevertheless, we do have some experimental models to indicate that it may not be too far-fetched. In setting up these models we attempted to produce a selective pharmacological agent which would elicit a type of response that could be predicted before the compound was synthesized and which had never before been called forth by a drug.

Tocopherol recommended itself as a metabolite for such a model, because in female mice a deficiency of this vitamin is apparent only in the pregnant individual, and is characterized by the resorptive interruption of pregnancy during the latter part of gestation. The deficiency is not fatal to the mother, and furthermore, does not produce readily demonstrable signs of disease in non-pregnant mice. A successful antagonistic structural analog of tocopherol should therefore produce resorptive interruption of pregnancy in mice, and should be without effect on non-pregnant individuals. $\alpha$-tocopherol quinone proved to be just such an agent (32). Its structural resemblance to tocopherol and also to vitamin K is shown in the next slide (figure 10). It is at the same time an analog of both vitamins. The data in the next slide (table 6) will show that daily oral ad-



$\alpha$-tocopherol $\alpha$-tocopherol quinone Vitamin $K_1$

$R = C_{16}H_{33}$

FIG. 10. Structures of $\alpha$-tocopherol quinone and vitamins E and K.

ministration, or a single parenteral dose caused the desired pharmacological effect. Curiously enough, its action was not reversed by tocopherol, but was by vitamin K. The size of the dose required of α-tocopherol quinone was so large that there is little possibility of it being of practical importance. Nevertheless, as an experimental model it may point the way to new and useful avenues of attack.

Now let us examine a piece of work which is just in progress at the present time, and which tends to show that inhibitory structural analogs of metabolites occur naturally in foods, and contribute to the production of disease. In order to do this I would like to talk very briefly about the etiology of pellagra. Our discovery in 1937 (1) that nicotinamide was the pellagra-preventative vitamin seemed to clear up the mystery of the cause of this disease, for the syndrome could be viewed as resulting from a lack of nicotinic acid in the diet. Indeed, it was shown that pellagragenic diets were low in this vitamin. Now, the eating of corn has been recognized for a long time as being intimately associated with the occurrence of pellagra. This has recently been forcefully pointed out by Aykroyd and Swaminathan (33), who showed that human diets containing corn and supplying 15 mg. of nicotinic acid per day were pellagragenic, while

TABLE 6

*Effect of dl-α-Tocopherol Quinone on Pregnant Mice*

| Oral dose | Intraperitoneal dose | Incidence of litters | Ave. size of litters |
|---|---|---|---|
| *mg. per day* | *mg.** | *per cent* | |
| 0 | 0 | 100 | 6 |
| 10 | 0 | 100 | 4 |
| 50 | 0 | 50 | 6 |
| 100 | 0 | 14 | 3 |
| 0 | 200 | 67 | 5 |
| 0 | 400 | 0 | |

* This was the total amount of material used per gestation.

corn-free rations yielding only 5 mg. of the vitamin never produced pellagra. Krehl, Tepley and Elvehjem (34) then clearly demonstrated that corn would reduce the rate of growth of rats, and that this action was overcome by nicotinic acid. They explained these observations largely on the basis of interference with the intestinal flora. However, some of the experiments which we had done with 3-acetylpyridine as a pellagragenic agent in mice led us to postulate that corn contains a structural analog of nicotinic acid which acts as a positive etiological factor in pellagra (25, 18). A substance has now been demonstrated in corn which causes pellagra-like manifestations in mice, and which is counteracted by nicotinic acid. This pellagragenic agent has been concentrated about 100,000 times, and appears to be a pyridine compound. The inference from this work therefore is that pellagra is a deficiency disease which results partly from a lack of sufficient nicotinic acid, but more especially from the action of an antagonistic agent in corn which competes with nicotinic acid, and thereby intensifies the deficiency.

Now that we have examined briefly some of the things which may be done with antagonistic analogs, we may wish to know what types of structural change applied to a metabolite will convert it into such an agent. I believe that certain generalizations can be made about the kinds of alteration which will do this (35). Most of the compounds described at the outset of this lecture as well as many others were predicted from these generalizations, and when they were finally synthesized, were found to have the forecasted biological action. Therefore, it may be justifiable to consider these generalizations briefly.

The first general type of structural change which will convert metabolites into inhibitors is the replacement of a carboxyl by some other more or less acidic grouping. This latter may be sulfonamide or sulfonic acid as in the case of the sulfonamide drugs and of thiopanic acid. On the other hand, it may be an aromatic ketone group as in the case of 3-acetylpyridine or phenyl pantothenone. These relationships are summarized in the next slide (figure 11).

The second general method of converting metabolites into inhibitors involves the exchange of one or more atoms in a ring system for some other atom. Examples of this type are shown in the next slide (figure 12). Here one can see that a sulfur atom may be replaced by two carbons as in the case of pyrithiamine; or carbons may be traded for nitrogens as in benzimidazole; or oxygens for carbons as in dicoumarol. A particularly effective method is to replace an atom in a ring with nothing at all, and thus arrive at an open chain-compound. Good examples of this may be seen in the desthiobiotin-derivatives with which du Vigneaud and his collaborators (36) have worked, or in $\alpha$-tocopherol quinone viewed as an analog of vitamin K as we have discussed earlier. Numerous other examples of this class are known from the works of many investigators but time has permitted the mention of only a few.

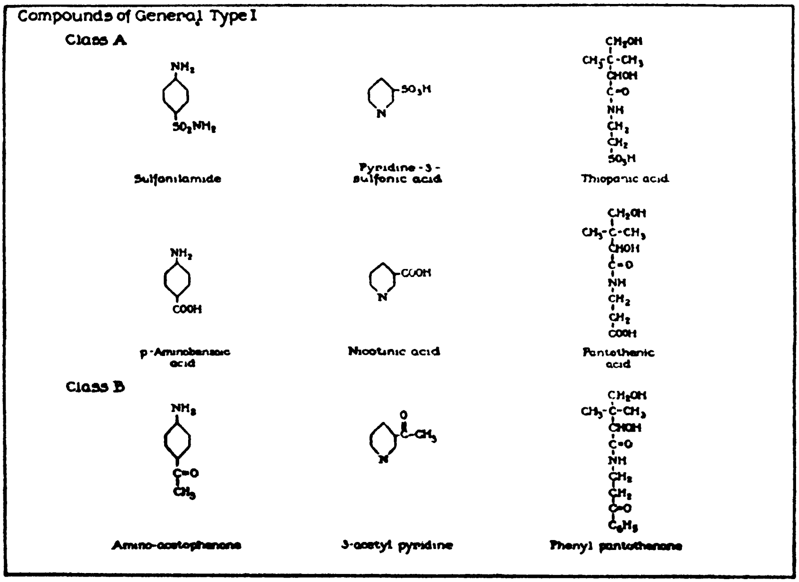
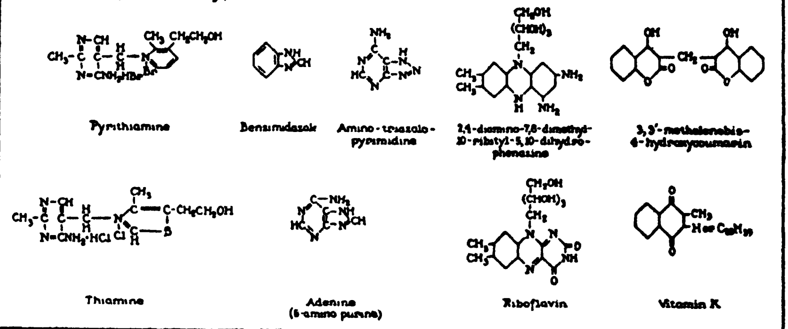


FIG. 11. Classification of analogs. Compounds of General Type I.

A third general method of realizing inhibitory structural analogs from metabolites is to replace alkyl side chains attached to aromatic nuclei with halogen-atoms. This is the type of alteration involved in the formation of 2,3-dichloro-naphthoquinone, an antagonist of vitamin K. Kuhn, Weygand and Möller (37) have produced competitors to riboflavin in this fashion, and there are other examples as well.

Finally, there are a number of miscellaneous types of structural alteration which have been found to result in the formation of antagonistic compounds when applied to metabolites. As work progresses, some of these may be found to be generally applicable. An example of these miscellaneous cases is the addition of an extra carbon to the chain of ascorbic acid with the resulting formation of glucoascorbic acid.

Compounds of General Type II


Pyrithiamine
Benzimidazole
Amino-triazolo-pyrimidine
2,4-diamino-7,8-dimethyl-10-ribityl-5,10-dihydro-phenazine
3,3'-methylenebis-4-hydroxycoumarin
Thiamine
Adenine (6-amino purine)
Riboflavin
Vitamin K

Compounds of General Type III

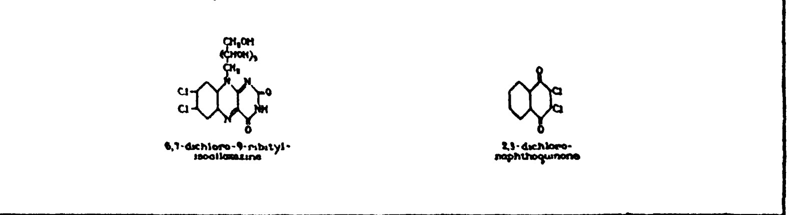

6,7-dichloro-9-ribityl-isoalloxazine
2,3-dichloro-naphthoquinone

FIG. 12. Classification of analogs. Compounds of General Types II and III.

In viewing these relationships of chemical constitution to biological activity, I want to emphasize that there is apparently no unique manner in which the structure of a metabolite must be altered in order to achieve an antagonist (35). Several inhibitory analogs of a single vitamin have been made by altering the metabolite in different ways. Interestingly enough, these varying antagonists of the same vitamin have certain qualitative differences in biological action.

For example, dicoumarol, 2,3-dichloro-naphthoquinone and α-tocopherol quinone are all analogs of vitamin K produced by divergent types of structural change. While all three can be shown to compete with the vitamin and thus to have certain biological effects in common, they have some very decided pharmacological differences. The fact that they do have certain biological properties in common may help us to understand a very old puzzle, namely, that substances of quite diverse structural changes may have similar action.

Despite the fact that diverse structural changes may be applied successfully to a given metabolite in order to produce an inhibitory analog, merely to alter the structure in any nonspecific fashion is not sufficient. Much testing of derivatives of the several vitamins has shown that many of these have no detectable antagonistic action.

In conclusion, it seems to me that we are only at the beginning. We have reconnoitered this new and virgin territory and have seen enough to make it seem worth while to explore it further.

## BIBLIOGRAPHY

1. Elvehjem, C. A., Madden, R. J., Strong, F. M., and Woolley, D. W., *J. Am. Chem. Soc.*, 1937, 59, 1767.
2. Woolley, D. W., Strong, F. M., Madden, R. J., and Elvehjem, C. A., *J. Biol. Chem.*, 1938, 124, 715.
3. Kuhn, R., *Angew. Chem.*, 1940, 53, 1.
4. Clark, A. J., *Hand. der Exp. Pharmakol.*, 1937, IV, Springer, Berlin, 190.
5. Woods, D. D., *Brit. J. Exp. Path.*, 1940, 21, 74.
6. Fildes, P., *Brit. J. Exp. Path.*, 1941, 22, 293.
7. McIlwain, H., *Brit. J. Exp. Path.*, 1940, 21, 136.
8. Snell, E. E., *J. Biol. Chem.*, 1941, 139, 975.
9. Woolley, D. W., and White, A. G. C., *Proc. Soc. Exp. Biol. and Med.*, 1943, 52, 106.
10. Woolley, D. W., and White, A. G. C., *J. Biol. Chem.*, 1943, 149, 285.
11. Robbins, W. J., *Proc. Nat. Acad. Sc.*, 1941, 27, 419.
12. Woolley, D. W., and White, A. G. C., *J. Exp. Med.*, 1943, 78, 489.

13. Woolley, D. W., and Krampitz, L. O., *J. Exp. Med.*, 1943, **78**, 333.
14. Woolley, D. W., *Fed. Proc.*, 1944, **3**, 97.
15. Goodman, L., Gilman, A., and Hart, N., *Fed. Proc.*, 1943, **2**, 80.
16. Woolley, D. W., *J. Biol. Chem.*, 1944, **152**, 225.
17. Woolley, D. W., *J. Biol. Chem.*, 1944, **154**, 31.
18. Woolley, D. W., *J. Biol. Chem.*, 1945, **157**, 455.
19. Auhagen, E., *Z. Phys. Chem.*, 1942, **274**, 48.
20. Woolley, D. W., and Collyer, M. L., *J. Biol. Chem.*, 1945, **159**, 263.
21. Ter Horst, W. P., and Felix, E. L., *Ind. and Eng. Chem.*, 1943, **35**, 1255.
22. Woolley, D. W., *Proc. Soc. Exp. Biol. and Med.*, 1945, **60**, 225.
23. Tamura, J. T., *J. Bact.*, 1944, **47**, 529.
24. Harris, J. S., and Kohn, J. I., *J. Pharmacol.*, 1941, **73**, 383.
25. Woolley, D. W., *J. Biol. Chem.*, 1946, **162**, 179.
26. Woolley, D. W., *J. Biol. Chem.*, in press.
27. Quastel, J. H., and Wooldridge, W. R., *Biochem. J.*, 1927, **21**, 1224.
28. Fildes, P., *Lancet*, 1940, **1**, 955.
29. Dittmer, K., and du Vigneaud, V., *Science*, 1944, **100**, 129.
30. McIlwain, H., and Hawking, F., *Lancet*, 1943, **1**, 449.
31. Overman, R. S., Field, J. B., Baumann, C. A., and Link, K. P., *J. Nutrition*, 1942, **23**, 589.
32. Woolley, D. W., *J. Biol. Chem.*, 1945, **159**, 59.
33. Aykroyd, W. R., and Swaminathan, M., *Indian J. Med. Res.*, 1940, **27**, 667.
34. Krehl, W. A., Tepley, L. J., and Elvehjem, C. A., *Science*, 1945, **101**, 283.
35. Woolley, D. W., *Science*, 1944, **100**, 579.
36. du Vigneaud, V., *Chem. Eng. News*, 1945, **23**, 623.
37. Kuhn, R., Weygand, F., and Möller, E. F., *Ber. Deutsch. Chem. Ges.*, 1943, **76**, 1044.

# INTER-RELATIONSHIPS BETWEEN GONADOTROPHIC AND SEX HORMONES[1]

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IT is a great pleasure indeed to pay another visit, my fourth one, to your country, and particularly to be privileged to address this group, an honor which I now share with the most outstanding scientists.

I wonder whether you free men living in a free country can fully appreciate how much it means to me to talk to you after having been shut off, like many others in my unfortunate country, from all free scientific life for a number of years. What little contact I have been able to maintain with a few scientific and personal friends not only exposed me but also them to grave danger.

You will, therefore, understand my reluctance and hesitation to present here observations and results obtained during these last years in the Netherlands in the field of sex endocrinology.

On former occasions I have pointed out that only a small part of the work discussed can be considered my own and that most of the results are the fruits of the efforts of my co-workers. This is even more true with regard to the work done during the last six years.

My report will deal mainly with investigations conducted at the Institute of Pharmacology of the University of Leyden, particularly by my friend, the head of the department, Professor S. E. de Jongh, and his assistant, Dr. Gaarenstroom (1), both of whom I was fortunate to have for many years on the staff of my pharmacologic laboratory at Amsterdam. For the past ten years these investigators have been working entirely independently but the relations between them and the co-workers, Dr. J. Freud and

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[1] **Lecture delivered April 18, 1946.**

Dr. Elizabeth Dingemanse, of the Amsterdam Institute have been very active and friendly. (It was a great satisfaction to me to see the honorary degree of Doctor of Medicine conferred upon Dr. Dingemanse in Amsterdam in January of this year in recognition of her chemical work which has contributed greatly to medical progress.)

When talking about us I want you to remember that I do not use this as a "pluralis majestatis" but that I want to give credit thereby to those of my co-workers whose share in these contributions so greatly overshadows mine.

One of the basic and most important discoveries in the field of sex hormonology was made independently and simultaneously by P. E. Smith (2) and by Aschheim and Zondek (3); they demonstrated the gonadotrophic activity of the anterior lobe of the pituitary and a similar property of human pregnancy urine.

It is likely that you over here are more familiar with the vast literature than we in Europe are but you will agree that it can be considered as established that the active principles from these two sources are not identical, and neither are the substances found later in the urine of castrates and climacteric women (4) and in pregnant mare serum (5). I hope you will not consider it immodest for me to say that I was probably the first to point out (during the Spring of 1930) that the similarity of effects obtained with pituitary extracts and with pregnancy urine does not prove anything about the chemical similarity, let alone about the identity of their active substances.

At the gynecologic congress in Frankfort the subject of "the anterior pituitary hormone in pregnancy urine" was ventilated by numerous speakers who, it seemed, hardly left a stone unturned, a fate which now has befallen the very building in which that meeting was held, due to the saturation bombing by the Allies. After at least twenty papers had been read on that subject, I asked during the discussion period the simple question, "Why are these substances referred to as pituitary substances?" This remark caused considerable excitement, particularly on the part of the principal lecturer, Zondek.

I went on to point out the possibility that the urinary substances might be quite different in nature, that they might not be derived from the pituitary and perhaps might not even act directly upon the gonads but only through the intermediary of the pituitary. It is intriguing that such ideas have recurred recently, in 1945, and, according to Guyénot (6) have some foundation.

Since P. E. Smith taught us the technic of performing hypophysectomy in rats two decades ago, which alone made a further analysis possible, there can be no doubt that urinary chorionic gonadotrophin can essentially act directly without the intermediary of the pituitary. It is now firmly established that the gonadotrophins from the four different sources are different not only quantitatively but also qualitatively. Moreover, it has been shown in our laboratory and in many others that gonadotrophins prepared from the pituitary of different species are not even identical (7). The pituitary extracts from horses differ in their properties from those obtained from sheep, the effect of the former for instance not being augmented by the simultaneous administration of chorionic gonadotrophin.[2]

I am pointing this out in the beginning in order to avoid the conclusion that observations made with the use of certain pituitary or chorionic gonadotrophins can be applied to all other gonadotrophins. It must also be remembered that the results I am going to report hold only for the species used, *i.e.*, rats or mice, and that any conclusion by analogy as to another species requires experimental confirmation, which fails us only too often.

But let us now come to our *main* subject: *the interrelationships between gonadotrophic and steroid sex hormones*—in other words their synergistic and antagonistic actions.

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[2] During the last meeting of the Committee on standardization of the late League of Nations held at Geneva in 1938, I tried in vain to defend the term "gonadotropin." However, in view of the current preference given the term "gonadotrophin," and especially after seeing Corner's discussion on this point (Endocr., 33: 405, 1943) I shall not only use it henceforth, but try to have it generally accepted in the Netherlands, since I think that unanimity is more important than etymological reasoning.

The subject which has been chosen is perhaps too narrow in scope and it may be necessary to include the action of these substances on the reciprocal sites of production as I have already done to a certain extent.

In 1926 (8) we demonstrated what we call the antimasculine effect. We showed that the development of the genitals of young male rats was stunted and that the size of the genitals regressed after the injection of estrogens. C. Hamburger (8a) in Copenhagen recently made the same observation in monkeys. Our views having been clouded by aprioristic ideas we assumed the existence of a direct antagonism of estrogens and the male gonads and thereby indirectly of the accessories. C. R. Moore (9) in Chicago demonstrated, however, that the effect is mediated by the pituitary and this has been confirmed by Lahr (10) in the Netherlands.

There can be no doubt about the inhibiting effect of estrogens on the gonadotrophic action of the pituitary as evidenced by the lack of development or maintenance of the testicles and the dependent organs; there can also be no question that such an inhibition of the gonadotrophic action is caused by androgens,[3] particularly by testosterone. This inhibition by the steroids is recognized in the morphologic picture of the ovary and of the testicle respectively and also by their functional state which is characterized by the small effect their own hormones exert upon the tubes, the uterus and the vagina on the one hand or the seminal vesicle, the prostate, etc., on the other. Of course, the inhibition of the accessories is observed only when androgens are administered to female or estrogens to male animals.

In both cases we are definitely dealing with an effect upon the sites of production of the gonadotrophins and not with an effect upon these substances themselves. This can be proved by the following observations.

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[3] The term ''androgen'' too is hardly justifiable and I fail to see why ''androgenic substance'' is preferred above ''male substance.'' However, for the sake of international unanimity I shall follow the practice of the majority.

First, if one administers gonadotrophins and sex hormones simultaneously to hypophysectomized animals, the action of the gonadotrophins is unaffected.

Second, the gonadotrophic activity of pituitaries pretreated with steroids (estrone, for example) is considerably weaker upon transplantation than that of untreated or oil-injected pituitary tissue.

TABLE 1

*Pretreatment of Male Rats with Testosterone Propionate (T. Pr.) Prevents Gonadotrophic Effect of Their Pituitaries When Implanted in Young Male Mice*

| Number of mice implanted with two pituitaries | Seminal vesicle weight | Testes weight | Pretreatment of donor rat daily | |
|---|---|---|---|---|
| | *mg.* | *mg.* | | |
| 5 | 3 | 12 | 0.5 mg. T. Pr. | castrated rats |
| 5 | 15 | 31 | Oil | |
| 5 | 3 | 13 | 0.5 mg. T. Pr. | normal rats |
| 5 | 13 | 33 | Oil | |
| 5 | 2 | 13 | No implant | |

Mice receiving pituitary implants from control rats show growth of testes and of seminal vesicles (regardless of whether the donor rats have been castrated or not). However, when pituitary implants are taken from rats pretreated with testosterone, the rate of growth of the gonads of the recipients is the same as though no implantation had been performed.

The cause of the decreased activity may lie in a diminished content, an impaired release, or a diminished production of hormone by such implanted tissue. What is new to us and probably also to you is the possibility of proving biologically the existence of two gonadotrophic substances, at least in the pituitary, the chemical demonstration of which under the designation of FSH and LH does not appear to us to have been absolutely successful up to this time.

We in Holland cannot entirely agree with these names because on the one hand they say too much and on the other hand they say too little. When separated entirely these substances probably can bring about neither complete follicular maturation nor luteinization. On the other hand each of them can produce more than mere maturation or luteinization.

Pituitary fractions with widely varying activities have been used and neither we, nor, I think, the majority of other investigators have been successful in obtaining with one such fraction luteinization without maturation and with the other maturation without luteinization. The fact that luteinization has been achieved by the combined action of FSH with quantities of LH so small as to be inactive by themselves proves that these names are misleading.

In order not to be misunderstood I want to emphasize that I do not doubt the existence of extracts of widely differing activities, particularly in view of the many American investigations, but I contest the description of such actions as "maturation" or "luteinization" and express doubts in the justification for the names allotted to such extracts. It would appear that the chemical separation has been successful (11–17), but at writing this I have seen only an abstract since the original literature was not yet accessible to us.

For this reason de Jongh proposed that the factor which acts particularly upon the interstitial cells and, therefore, brings about luteinization more easily after a certain degree of maturation has taken place be designated as GI[4] (i.e., gonadotrophin affecting the interstitium). He also suggested that the other factor which acts perhaps upon the ovocyte and definitely upon the "ovariocytes" which are in epithelial arrangement (?) be designated as GE (i.e., gonadotrophin affecting the epithelium).[5]

[4] The term LH has been replaced in the scientific language during the past years by ICSH on the same grounds which made de Jongh coin GI. This latter term will hardly be acceptable in America because it has become a very widely known abbreviation in two entirely different contexts.

[5] Freud applies the term ovariocytes, in a way which I consider very appropriate, that is, to all cells of the ovary with the exception of the egg cells, connective tissue cells, cells of the blood vessels, the nerve-ganglia and nerve sheaths.

I will give you another example (18, 19) to show why these names are superior to, and why they presume less than, the older designations.

The administration of castrate urine brings about follicular maturation and if an amount 10 to 20 times that needed for follicle maturation is injected luteinization results. A small amount of pregnancy urine alone has almost no effect. However, one obtains an extraordinarily strong follicular maturation and luteinization if a small amount of castrate urine together with the formerly almost inactive quantity of pregnancy urine is used. We are, therefore, dealing here with something very different from a simple additive effect and the same holds true for the chemically separated pituitary fractions as for the naturally occurring fractions in these urines.

Greep (20) observed that highly purified FSH, while it causes enlargement of the follicle, does not induce production of estrone, an effect formerly attributed to this fraction and previously considered a function closely connected with the morphological changes of maturation.

In view of the uncertainties concerning the limit of action of the different factors and the fact that the incomplete chemical separation has permitted doubt as to the existence of two different entities it is certainly of interest to have presented proof of the qualitative difference in action of these two substances in vivo, which is not necessarily the case even if the substances differ chemically.

### BIOLOGICAL PROOF OF THE DUALITY

My colleagues de Jongh and Gaarenstroom (21) in Leyden have also succeeded in presenting such a biological proof.

It is well known that hypophysectomy is followed by a definite and progressive atrophy of the testicles. This can be overcome by the implantation of pituitaries. That is, atrophic testes can be brought back to a normal state and even to the point at which growth occurs.

Such growth cannot be induced by chorionic gonadotrophin which in its other effects resembles most those of the GI (ICSH).

Chorionic gonadotrophin, which will be discussed later on in more detail, can only prevent the progress of atrophy; thus, it preserves the existing state or at the most induces under certain conditions a very small degree of growth. However, the effect of pituitary implants is materially increased if chorionic gonadotrophin is administered simultaneously.

TABLE 2

*Testes of Hypophysectomized Rats after Implantation of Pituitaries from Pretreated Rats*

| Number of animals | Implanted with | Treated daily for 1 week with | Testis weight | | Ave. % difference with standard deviation |
|---|---|---|---|---|---|
| | | | Before implantation | After implantation | |
| | | | *mg.* | *mg.* | |
| 8 | | | 161 | 68 | −60 ± 1 |
| 6 | 2 half normal pituitaries | | 188 | 188 | − 2 ± 7 |
| 9 | 2 half "estrone-pituitaries" | | 190 | 111 | −42 ± 3 |
| 7 | | 5 I.U. of chorionic gonadotrophin (GI) | 106 | 119 | +16 ± 9 |
| 6 | 2 half normal pituitaries | 5 I.U. of chorionic gonadotrophin (GI) | 98 | 166 | +80 ± 17 |
| 8 | 2 half "estrone-pituitaries" | 5 I.U. of chorionic gonadotrophin (GI) | 100 | 171 | +86 ± 16 |
| 11 | 1 half normal pituitary | 5 I.U. of chorionic gonadotrophin | 120 | 165 | +42 ± 7 |
| 8 | 1 half "estrone-pituitary" | 5 I.U. of chorionic gonadotrophin | 120 | 158 | +40 ± 10 |

The gonadotrophic effect is distinctly decreased if hypophysectomized rats are implanted with pituitaries obtained from estrone-treated rather than from normal animals. The "estrone-treatment" consists of injections of 0.2 to 0.5 milligrams of estrone daily for a period of two weeks before the pituitaries are transplanted. At the time of the implantation of the pituitaries one testicle of the rat is removed for comparison with the other one, extirpated one week later.

Hypophysectomy causes a 60% loss of weight of the testes within one week (line 1). The implantation of one or two half normal pituitaries[6] prevents atrophy (line 2). There is no loss of weight but one or two half "estrone-pituitaries" are unable to prevent such a loss and still permit a drop of weight of approximately 40% (line 3).

The injection of an ample dose of chorionic gonadotrophin[7] into a hypophysectomized rat not only abolishes the atrophy but induces a small growth (line 4). If one whole or two half[6] "normal" pituitaries are implanted the resulting growth amounts to 80% (line 5), and if one half a pituitary only is used, the testicular weight increase amounts to approximately 40% (line 7). The "normal" pituitaries used for implantation are taken from rats injected with oil instead of with estrone benzoate in oil.

Implants of "estrone-pituitaries" instead of normal pituitaries are, however, no less effective if chorionic gonadotrophin is injected simultaneously (line 6). Therefore, it is the lack of gonadotrophic activity caused by the treatment with estrone which is made up by the chorionic gonadotrophin.

It is of decisive importance, however, that even large doses of chorionic gonadotrophin cannot replace half a pituitary. The effect obtained by implantation of only one-half of a pituitary in spite of a large dose of chorionic gonadotrophin lags behind that obtained with two half pituitaries (compare lines 7 and 8 with lines 5 and 6).

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[6] Two half pituitaries were used in these experiments to avoid the criticism that half a pituitary with an exposed cut surface might contain less gonadotrophins than half a pituitary in the uncut gland.

[7] Pregnyl 'Organon.'

The following equations are probably the shortest condensation of the above:

$$\begin{aligned} \text{``estrone pituitary''} &< \text{normal pituitary} \\ \tfrac{1}{2}\ \text{pituitary} &< 1\ \text{pituitary} \\ \text{``estrone pituitary''} + \text{GI} &= \text{normal pituitary} \\ \tfrac{1}{2}\ \text{pituitary} + \text{GI} &< 1\ \text{pituitary} \end{aligned}$$

*Conclusion:* Much GI overcomes the damage caused by estrogens but not by loss of ½ pituitary. Therefore, that half pituitary must contain something other than GI (perhaps GE).

### PRACTICAL APPLICATION OF THE ANTIGONADOTROPHIC EFFECTS OF THE STEROIDS

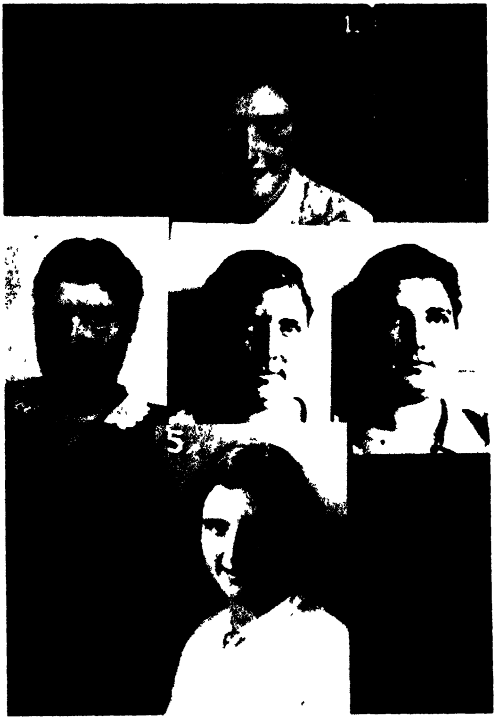
The antagonistic effects have so far been considered only, so to speak, in reverse direction: from the gonads upon the pituitary. I wish here to touch only upon the fact that these effects can have practical therapeutical importance. For instance, the treatment of Cushing's syndrome which we (22) undertook successfully for the first time 12 years ago in Holland and which has been repeated by others with similar results.

A girl of approximately 20 years of age came to my office with a fully developed Cushing's syndrome (figure 2). She exhibited the typical full-moon face, striae, amenorrhea, high blood-sugar levels, raised arterial blood pressure, and increased hemoglobin, and the erythrocyte count was above 6.5 million per mm$^3$. There was no hirsutism. The first picture (figure 1) shows the patient approximately two years before I saw her for the first time. She received comparatively large doses of estradiol benzoate[8] (50,000–100,000 I.B.U. per day) for two periods of 14 days each, separated by 3½ weeks without any treatment. After the third day of therapy the number of erythrocytes, the hemoglobin and the fasting blood sugar decreased. Menstruation was re-established after six weeks and continued at regular intervals for five additional periods. The facial expression improved (figures 3 and 4) and the striae diminished The blood pressure was not affected. The last picture (figure 5) shows the patient 1½ years after the

[8] Dimenformon Benzoate 'Organon.'

beginning of treatment. By that time she had married and has since become the mother of, I believe, three children.

Recently we have seen another case in Amsterdam which was distinctly favorably influenced by estrone administration so that we could avoid irradiation therapy and certainly surgery.



Cushing's Syndrome Cured by High Estrogen Treatment
Fig. 1. Approximately two years prior to first visit.
Fig. 2. At first visit in 1934.
Fig. 3. One month later.
Fig. 4. Another two months later.
Fig. 5. Approximately 1½ years after beginning of therapy.

I would like to mention the recent successful replacement of estrone by testosterone for the inhibition of the pituitary (23).

## GONADOTROPHINS AND STEROIDS WITHIN THE GONADS

### A. *In the Testes*

I would like to discuss particularly the interaction of the gonadotrophins with the steroids *within* the gonads themselves.

I repeat that these investigations are not mine but those of de Jongh and his co-workers, particularly Gaarenstroom (1). The direct influence of the steroids, i.e., of estrone, progesterone and testosterone, upon their own sites of production, the ovaries and the testicles, was opposed by the justified prejudices that these steroids can in general exert only an inhibitive influence.

For instance, the function of the Islands of Langerhans also was supposed to be depressed by insulin and the function of the thyroid by thyroxine, etc. In a similar way the female substances such as the estrogens were supposed to exert only a suppressive influence, if any.

a. *Androgens* (*testosterone*). Similar relationships as those which obtain for the effect of thyroxin upon the thyroid or estrogens upon the ovary ought to prevail between testosterone and the testis. In the last analysis this is actually the case. Testosterone does not have a stimulating action on the tissues which produce it but upon those which surround it and which are situated in the "same organ only according to our morphological classification." Nature, however, does not care about the lines we draw roughly and arbitrarily. What we call an "organ" because it can be easily differentiated anatomically from other structures can consist of parts with very different functions (for example, the pituitary and the adrenals) and on the other hand tissues lying far apart from each other (such as scattered islands of chromaffin tissue) can function as one organ. However, the close proximity of functionally and morphologically different structures is of particularly great importance—for example, the position of the pituitary as a brain adnexa, and probably also the juxtaposition of the pituitary lobes and that of the adrenal cortex and medulla. This is reminiscent of Zoroaster's words (24): "There is more sense in your body than in your supreme wisdom." Gigon and Buchs (24a) recently pointed out interesting relations between proximate glands and speak about a "Koordinationsregel von Drüsenpaaren."

*Maintenance.* We have confirmed in Holland the observation previously made by others that not only chorionic gonadotrophin but also testosterone will maintain spermatogenesis which ceases regularly (25–28) following hypophysectomy. The size of the testicle remains unchanged and growth occurs only if the administration of testosterone is started within the first few days after hypophysectomy because remnants of the pituitary hormone are still in circulation at that time. Half a milligram of testosterone per day has a maintaining effect upon the tubules of rats both morphologically and functionally. Moreover testosterone augments the restoring effect of pituitary gonadotrophins[9] upon spermatogenesis in cases of testicular atrophy caused by hypophysectomy. In this connection it is interesting that Leathem and Brent (28a) have been able to maintain spermatogenesis in hypophysectomized rats with pregnenolone.

The following, however, is interesting. A dose adequate to maintain a testis weighing one gram can also only maintain a 10 times smaller testis of only 100 milligram weight but cannot induce it to grow. This "maintenance" differs from growth. Ac-

TABLE 3

*Maintenance of Testis of Hypophysectomized Rats by Testosterone Propionate (T. Pr.) for Two and Three-week Periods (3 Groups of Different Ages)*

| Number of rats | Weight before operation | Testis weight | | Daily treatment |
|---|---|---|---|---|
| | | Beginning | At autopsy | |
| | *gm.* | *mg.* | *mg.* | |
| 3 | 46 | 132 | 119 | 0.5 mg. T. Pr. |
| 6 | 46 | 139 | 60 | Oil |
| 4 | 69 | 164 | 141 | 0.5 mg. T. Pr. |
| 7 | 162 | 1104 | 887 | 0.6 mg. T. Pr. |
| 10 | 170 | 1060 | 175 | Oil |

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[9] Ambinon 'Organon.'

cording to Gaarenstroom and de Jongh (29) it is an effect "sui generis." The essential facts can be seen from table 3 which is a condensation of many experiments.

Testosterone can not only prevent the progress of the atrophy which occurs after hypophysectomy but, as I have said before, it can overcome the inhibition of growth within the testes which is caused by estrone. In young animals this compound can thereby even cause a certain degree of growth.

TABLE 4

*Testosterone Propionate (T. Pr.) Prevents Testis Atrophy Caused by Estrone in Rats*

| Number of rats | Treatment | Weight | | Testes weight | Daily treatment | | |
|---|---|---|---|---|---|---|---|
| | | Beginning | At autopsy | | Estrone | Oil | T. Pr. |
| | *days* | *gm.* | *gm.* | *mg.* | γ | | *mg.* |
| 10 | 14 | 32 | 66 | 550 | | + | |
| 10 | 14 | 32 | 56 | 580 | | + | 0.5 |
| 10 | 14 | 32 | 59 | 145 | 50 | + | |
| 5 | 14 | 32 | 61 | 270 | 50 | + | 0.1 |
| 10 | 14 | 32 | 60 | 550 | 50 | + | 0.5 |
| 3 | 14 | 85 | 147 | 1700 | | + | |
| 3 | 14 | 83 | 141 | 1475 | | + | 0.5 |
| 3 | 14 | 82 | 138 | 480 | 20 | + | |
| 4 | 14 | 82 | 138 | 1960 | 20 | + | 0.5 |
| 4 | 24 | 55 | 121 | 1270 | | + | |
| 4 | 24 | 57 | 129 | 1290 | | + | 0.5 |
| 4 | 24 | 54 | 110 | 220 | 20 | + | |
| 4 | 24 | 57 | 116 | 1270 | 20 | + | 0.5 |
| 5 | 28 | | 174 | 1740 | | | |
| 10 | 28 | | 176 | 1880 | | + | 0.5 |

This effect of testosterone which thus far has been unknown is not caused by the pituitary which one might suspect to be stimulated by testosterone but the contrary is true, testosterone inhibits the pituitary. (The pituitaries of rats treated with testosterone show a decreased gonadotrophic activity when used as transplants.) (See table 1, page 220.) Furthermore, testosterone does not prevent the action of estrone upon the pituitary, since the pituitaries of animals treated simultaneously with estrone and testosterone show the usual decreased gonadotrophic activity as again evidenced by transplantation experiments.[10]

This decreased weight of the seminal vesicles is actually caused by a diminution in the production of gonadotrophins. This drop might be explained, in the light of the investigations by Westman and Jacobsohn (30), by an abnormally high output of the secretion before transplantation, since these investigators found separate innervations for the production and for the release of the pituitary hormones. On the other hand, experiments with parabiosis (31, 32) leave no doubt that testosterone actually inhibits the production of the gonadotrophin.

TABLE 5

*Testosterone Propionate (T. Pr.) Does Not Prevent the Inhibiting Effect of Estrone on the Pituitary as Ascertained by Implantation in Young Male Mice*

| Number of mice | Pretreatment of rats for 16 days with | | | Weight | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | At implantation | | | At autopsy | |
| | Estrone | Oil | T. Pr. | Rat | Rat pituitary | Mouse | Mouse | Seminal vesicle |
| | γ | | *mg.* | *gm.* | *mg.* | *gm.* | *gm.* | *mg.* |
| 3 | | + | | 99 | 5 | 5.5 | 8.0 | 12.0 |
| 5 | 20 | + | | 106 | 7 | 6.0 | 8.0 | 3.5 |
| 5 | | + | 0.5 | 117 | 4 | 5.5 | 7.5 | 3.0 |
| 3 | 20 | + | 0.5 | 110 | 5 | 5.5 | 7.0 | 3.0 |

In spite of the fact that the pituitary was inhibited, animals treated with both testosterone and estrone have testes and accessory organs of normal size or even slightly larger than the size before the injection of the above steroids (33).

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[10] According to Gaarenstroom there exists, however, a certain antagonistic influence through a direct effect on the pituitaries since an increase in the size of these glands, even to the extent of tumor production, caused by estrone can be prevented by testosterone.

These facts must be explained on the basis of a direct, stimulating effect of testosterone upon the testes. This action seems to consist of a maintaining effect upon the tubules (which contribute most to the size of the organ). Besides, the quantities of GE (FSH) produced (even in the pituitary inhibited by estrone and testosterone) suffice for the stimulation of the size, since we have seen above that the inhibition of the pituitary does not interfere at all with the production of GE.

The same results as those observed with testosterone can also be obtained through administration of the chorionic gonadotrophin. The lacking GI can therefore be substituted by testosterone. Histologic pictures of testes help to strengthen this concept. The testes of rats treated with estrone alone are small, and show atrophic tubules and only very small groups of interstitial tissue. Testes of rats treated with large doses of testosterone only are of normal size and contain well-developed tubules, but almost no interstitium. A similar size of testes with a similar histologic appearance is obtained in the rat when both these steroids are used together. Such testes differ from normal ones only in that their interstitial spaces are empty.

I have emphasized in the foregoing that the doses of testosterone used in the rats must be large because smaller doses produce effects indicating pituitary inhibition and are inadequate for maintaining the size of the testes.

I should like to mention here the experiments of Nelson (34) who succeeded in producing growth of the testes in hypophysectomized animals by injecting cortical extracts and on the other hand results obtained by Gaarenstroom and de Jongh (35) who failed either to maintain the ovarian size and structure of the ovary or to prevent its atrophy after hypophysectomy by injections of testosterone.

The main fact is that the damage which is done to the gonadotrophic function of the pituitary by the injection of estrone and which is responsible for the so-called antimasculine effect of this hormone cannot be observed after large doses of testosterone. It would appear that testosterone exerts a maintaining effect upon the testicular tubules.

Is it then too farfetched to explain the similar effect of the chorionic gonadotrophin upon the testes by its ability to stimulate the production of testosterone which, in turn, exerts a local effect?

The effect on the cells of the testicular interstitium has long been considered one of the most important, if not the most important, actions of the chorionic gonadotrophin. The interstitium thus becomes the site of newly produced male hormone. As a matter of fact, treatment of animals with GI (chorionic gonadotrophin) preserves not only the tubules but also the interstitium in a good condition. It is therefore possible to differentiate at a glance between treatment with testosterone on the one hand and with chorionic gonadotrophin on the other by examining the tissue microscopically. The rats which received chorionic gonadotrophin show well-maintained tubules and interstitium while in those treated with testosterone only the tubules are normal.

TABLE 6

*Hypophysectomized Rats Treated for 2 Weeks Starting Immediately After Operation*

| Number of animals | Ave. weight | | Ave. testis weight | | % difference | Daily treatment |
|---|---|---|---|---|---|---|
| | Beginning | At autopsy | Beginning | At autopsy | | |
| | *gm.* | *gm.* | *mg.* | *mg.* | | |
| 3 | 46 | 55 | 139 | 60 | −57 | 0.1 cc. oil |
| 6 | 46 | 56 | 132 | 119 | −10 | ½ mg. T. Pr. |
| 6 | 48 | 54 | 153 | 137 | −11 | ¼ R.U. pituitary gonadotrophin* |
| 6 | 47 | 54 | 131 | 232 | +77 | ½ mg. T. Pr. + ¼ R.U. pituitary gonadotrophin* |

* Ambinon 'Organon.'

The general conclusion has therefore been drawn that testosterone exerts no stimulating effect locally on the site of its production.

I will mention only the main results of the experiments which led to the conclusion that GI exerts its stimulating effects exclusively through the production of testosterone. It is well known that the effect of pituitary extracts upon the testes of hypophysectomized rats is increased considerably by the addition of GI (chorionic gonadotrophin) (36, 37, 38, 39). Both GI alone and testosterone alone can maintain the structure of the testis in exactly the condition it was in at the time of hypophysectomy.

Testosterone can also bring about the augmenting effect, for which GI is known, when added to pituitary extracts.

At the time of hypophysectomy one testis is removed, the other being removed at the end of the experiment two weeks later. Daily injections of oil, pituitary extract,[11] testosterone propionate, or a combination of the last two were given starting immediately after hypophysectomy. The results are listed in table 6. They show that the testicular atrophy in young hypophysectomized rats was not so great when small doses of pituitary extract or testosterone were given as when no hormones were injected, and that a combination of these two hormones resulted in a definite growth of the testis. This proves that testosterone has the same augmenting properties previously demonstrated by others for GI. On the other hand, GI (chorionic gonadotrophin[12]) has hardly any more effect than testosterone.

Table 7 shows (lines 2 and 3, 7 and 8) that adding chorionic gonadotrophin to a combination of pituitary gonadotrophin and testosterone has little effect. This does not mean that the maximum effect has already been reached; on the contrary an increase of the dose of testosterone yields an increase in weight.

During normal growth, too, the development of the tubules is initiated and stimulated by GE while GI causes the production of testosterone in the interstitium. The testosterone maintains the developed tubules. In the light of this, the tubules are to be classified with the accessory glands and sex organs which are dependent on the hormone produced in the primary organ of the

[11] **Ambinon 'Organon.'**

[12] **Pregnyl 'Organon.'**

## TABLE 7

*Immature Hypophysectomized Rats Treated with Hormones Beginning with the First Day after Operation*

| Line No. | Number of animals | Daily treatment | Ave. body weight |  | Ave. testis weight |  | % difference |
|---|---|---|---|---|---|---|---|
|  |  |  | Before treatment | After treatment | Before treatment | After treatment |  |
|  |  |  | *gm.* | *gm.* | *gm.* | *gm.* |  |
|  |  | *For 2 weeks* |  |  |  |  |  |
| 1 | 6 | 0.25 R.U. pituitary gonadotrophin* | 49 | 54 | 0.15 | 0.14 | − 7 |
| 2 | 6 | 0.25 R.U. pituitary gonadotrophin*<br>+ 0.5 mg. testosterone propionate | 47 | 54 | 0.13 | 0.23 | + 77 |
| 3 | 5 | 0.25 R.U. pituitary gonadotrophin*<br>+ 0.5 mg. testosterone propionate<br>+ 5 I.U. chorionic gonadotrophin† | 49 | 60 | 0.15 | 0.22 | + 47 |
| 4 | 8 | 5 R.U. pituitary gonadotrophin* | 49 | 59 | 0.15 | 0.20 | + 33 |
| 5 | 8 | 5 R.U. pituitary gonadotrophin*<br>+ 5 I.U. chorionic gonadotrophin† | 48 | 56 | 0.15 | 0.35 | + 133 |
| 6 | 5 | 5 R.U. pituitary gonadotrophin*<br>+ 0.5 mg. testosterone propionate | 50 | 60 | 0.15 | 0.33 | + 120 |
|  |  | *For 10 days* |  |  |  |  |  |
| 7 | 7 | 0.25 R.U. pituitary gonadotrophin*<br>+ 1 mg. testosterone propionate | 74 | 78 | 0.08 | 0.18 | + 125 |
| 8 | 8 | 0.25 R.U. pituitary gonadotrophin*<br>+ 1 mg. testosterone propionate<br>+ 5 I.U. chorionic gonadotrophin† | 72 | 76 | 0.07 | 0.19 | + 170 |
| 9 | 3 | 5 R.U. pituitary gonadotrophin*<br>+ 1 mg. testosterone propionate | 65 | 67 | 0.06 | 0.23 | + 287 |

* Ambinon 'Organon.'

gonad. The close vicinity of the site of production of testosterone ensures the effect upon the tubules.

Nevertheless, I wish to point out that it is necessary to exercise caution in making such conclusions. Obviously analytical experiments cannot teach us more than the possibility that development, produced with certain substances under arranged experimental conditions, may take place, we cannot prove yet that it does take place under normal conditions.

It is even more likely that the contrary is the case, which would mean in this connection that GI, too, may be able to exert a direct influence on the tubules. Experiments in sparrows lend support to this contention since we cannot speak about any interstitium in these birds and the tubules of animals during the winter season have been noticed to develop remarkably after injections of chorionic gonadotrophin[13] (de Fremery (39a)).

It must be stated furthermore that facts which have been proved conclusively in rats do not necessarily hold for other species. The same results have so far been obtained in mice (40) but not in guinea pigs (41) nor, at least in the early experiments, in monkeys, which is of greater interest to us. Yet the possibility cannot be excluded that the doses used in the latter experiments may have been too small. The most recent experiments by P. E. Smith (42), in which he showed the great importance of the local activity of some substances, lend support to this conclusion. He obtained excellent, though localized, spermatogenesis in the region in which pellets of testosterone propionate had been implanted into the testicle (42a). The importance which the dose of testosterone has locally upon spermatogenesis had previously been demonstrated by Dvoskin (43) in interesting experiments. On the other hand the most recent experiments of Masson (43a) show that many substances other than testosterone and the above-mentioned pregnenolone, some of which are steroids of very low androgenic activities, can exert a definite maintenance effect after hypophysectomy. I regret that time does not permit me to tell you about de Jongh's interesting explanation of the mechanism of this effect.

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[13] Pregnyl 'Organon.'

b. *Estrogens.* In the past the estrogenic substances were never observed to exert any direct effect upon the testis. In view of the aprioristic ideas we had 20 years ago we were naturally highly surprised in those days to find estrogen in the rat's most masculine organ, the testis (44, 44a). Since that time large quantities of estrogens have been found in the testes of stallions (which to date are the richest source of all biological estrone-containing materials). Only a few weeks ago Professor Ruzicka told me that he had succeeded in isolating estrone from pig testes. Whether or not the presence of estrone in testes is of physiologic importance we do not know yet. I only want to remind you of the so-called "pacemaker" effect (Freud (44b)) which consists in an augmentation of the growth of the seminal vesicles induced by testosterone and particularly by androsterone when small amounts of estrone are administered simultaneously.

### B. *In the Ovary*

We now come to the direct effect of steroid substances upon the ovary. There can be no doubt about the existence of this effect. As a matter of fact we have to consider not only the effect of female hormones but also that of testosterone. There are even good reasons to presume that such effects both of estrogens and male substances occur normally. So far it has not been possible to identify the chemical nature of such substances. Due to the limitation of time at my disposal I cannot present the proof in detail and I can give you only a sketch of our views, i.e., particularly those of the laboratory in Leyden.

### CONSIDERATION OF DIFFERENCES IN EXPERIMENTAL TECHNIQUES

Some progress has been derived and more will come forth. By differentiating sharply between results obtained on the one hand in normal, and on the other hand, in hypophysectomized animals in which latter case one has to consider carefully the time which has elapsed since hypophysectomy because one has to take into account the presence of endogenous pituitary substances during the first days, particularly during the first 24 hours, following operation. For such reasons we must also distinguish clearly between adult and non-adult animals and among the latter ones, furthermore, between completely immature and so-called infantile animals.

It may enable you to judge future publications better if I emphasize that every extrapolation, that is, transfer of experiences obtained in one species of a certain age made under specific conditions, can cause dangerous errors if applied to a different species and even to the same when the experimental conditions are slightly changed.

a. What is the effect of estrogenic substances? We know the so-called Collip (45) and Hohlweg (46) effect and its explanation. According to these authors large doses of estrone have a strong gonadotrophic effect (47) in young normal rats of 20–30 grams. Upon closer analysis the following effects can be differentiated:

1. Increase of the weight of the ovaries.
2. Increase in follicles without a cavity in very young animals and of follicles with some cavities in slightly older animals.
3. Development of corpora lutea though not regularly in such slightly older animals.
4. By extending the treatment to three weeks David, Freud and Uyldert (48) in our laboratories obtained a prolongation of the lifetime of corpora lutea and their change in the direction of corpora lutea of pregnancy and even complete transformation into such.

### EXPLANATION CONTRARY TO HOHLWEG

Let us call Number 5 the Collip effect proper which consists in the increase in size of the ovary, its maturation, and the formation of corpora lutea if treatment with chorionic gonadotrophins is preceded by estrone administration (45). The situation arising after the injection of estrogens into normal animals having pituitaries is actually quite complicated and the results became the more conflicting the more such experiments were performed. These investigations more than anything else have shown the great importance in every respect of the quantity factor: the dependence of the results upon the dosage, the type of estrogen used, the distribution of the doses over different intervals and different periods of time.

Most surprising were the effects mentioned first which were published more than 12 years ago by Hohlweg (46). A single injection of 500 R.U. (about 0.3 milligram) of estrone into the rat caused first, the formation of corpora lutea and only secondly, an increase in the size of the pituitary.

I do not want to elaborate on the extensive literature on this subject since the five principal points concerning the ovary have already been mentioned. I only want to point out that Hohlweg and others consider the changes in the ovary as dependent on the increase in size of the pituitary and they presume that this reflects an increased function or rather a larger release of the secretion of this gland (49). While it is not impossible that such a relation exists, it has been overlooked—and this is important for our consideration—that there may also exist a direct effect of the estrogens upon the gonad aside from the indirect influence of the pituitary. Today we can state that it has been proved that such a direct influence exists.

Our colleagues in Leyden (50) have obtained such changes in the ovaries of hypophysectomized rats after a single injection of 1 milligram of estradiol benzoate. Such ovaries were almost twice the size and weight of those of untreated four-week-old control rats. The difficult and cumbersome counts of Paesi proved the point. He found that the increase in size of the ovary is mainly the result of an increase in medium-sized follicles without cavities and therefore is not caused by a prolongation of the life period of already existing follicles. Of course the existence of such a life-prolonging effect upon follicles cannot be ruled out and it is possible that estrone may have a "maintenance" effect on the follicles analogous to that of testosterone on the tubules. The large number of mitoses in the granulosa shows, furthermore, that estrone has also a growth-stimulating effect.

It is also of importance that it was observed in the above experiments that the ovum continues to exist, that no atretic follicles are formed and that no luteinization occurs in these young animals. Such changes would have to be preceded by a hypertrophy of the theca which was absent in these experiments. This effect of estrone becomes particularly evident when subthreshold doses of gonadotrophins (for instance, pituitary extract) which are too small to have an effect are given or gonadotrophins which give only an incomplete reaction (chorionic gonadotrophin). In both cases the Leyden investigators succeeded in demonstrating the strong effect of estrone on the ovary. When stilbestrol was given to normal animals receiving large doses of chorionic gonadotrophin (which lacks GE) they obtained the expected increase in activity of this synthetic estrogen. Thereupon they treated hypophysectomized animals with chorionic gonadotrophin plus estradiol benzoate and they obtained follicles with definite cavities and hypertrophy of the theca. The theca never responds to either estrone alone or chorionic gonadotrophin alone but does give a good response to the simultaneous administration of both these hormones. I shall tell you how de Jongh interprets the mechanism of this effect.

### SYNERGISM OF ESTROGENS AND GONADOTROPHINS

The above-mentioned transformation into corpora lutea of pregnancy by prolonged administration (3 weeks) of estrogens (among which also the rather poorly active equilinine (51)) can be considered as proof of a synergistic action of the estrogens with pituitary gonadotrophins (52)[14] since the pituitary has certainly been damaged following prolonged treatment and, therefore, releases diminished amounts of gonadotrophins. From their observations that the reproductive organs (uterus, vagina) undergo progestational transformation and that the mammary gland develops, Uyldert *et al.* (55) concluded that the morphological changes in the corpus luteum are paralleled by a functional change (production of progesterone) in the corpus luteum (Uyldert 56)).

I should like to insert here a remark concerning the difference in responses of animals at different ages.

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[14] A number of papers (53–54a) of which only abstracts have been accessible to us point in the direction of such a synergism.

### DIFFERENCES IN THE SENSITIVITY OF THE OVARY

It is of general importance and often overlooked that a stronger reaction must not necessarily be caused by an increased release of another substance (the above case of more gonadotrophins) but that the reactivity and sensitivity of the responding organ is a different one.

We believe that the sensitivity of the growing animal is increased as compared to that of very young ones and it is possible that the gradual increase in estrone plays a role. This is probable in view of experiments in which one-week-old rats showed no effect whatsoever following the injection of chorionic gonadotrophin. The gonadotrophic activity of the pituitary as measured by their effect following transplantation does not increase according to Clark (57) in older animals as compared to those of younger ones. Thus it is not the pituitary but the ovary which changes during the course of the development of the animal.

b. Do we also know the effects of male hormone within the ovary?

### TESTOSTERONE AS A LUTEINIZING FACTOR

Such an effect has been clearly demonstrated by de Jongh and Gaarenstroom. The ovaries from the infantile animal were brought into a very far advanced stage of maturation by pretreatment with a combination of pituitary and chorionic gonadotrophins. Such ovaries weighed approximately 65 mg. instead of 7–11 mg. and showed large follicles and corpora lutea.

The pretreated rats were hypophysectomized and one ovary was removed. Ten days later the other ovary was removed and showed a far-advanced atrophy in untreated animals. Such ovaries weigh only 30 mg. which represents a loss of weight of approximately 55%. When either the pituitary gonadotrophin or chorionic gonadotrophin are administered separately the loss of weight is smaller amounting to approximately 43%. Following the simultaneous administration of both the chorionic and pituitary gonadotrophin practically no loss of weight (4%) is obtained. A combination of the pituitary extract with estrone

## TABLE 8

*Female Rats Pretreated with Pituitary* and Chorionic† Gonadotrophin, Hypophysectomized and Unilaterally Castrated, Injected for Ten Days*

| Number of animals | Daily treatment | Ovarian weight | | % decrease | Number of large corpora lutea (relative value) | | % decrease |
|---|---|---|---|---|---|---|---|
| | | Before treatment | After treatment | | Before treatment | After treatment | |
| | | *mg.* | *mg.* | | | | |
| 9 | None | 68 | 30 | 56 | 109 | 57 | 48 |
| 9 | 50 γ estrone | 70 | 31 | 56 | 97 | 50 | 48 |
| 13 | 5 R.U. pituitary gonadotrophin* | 62 | 35 | 44 | 93 | 45 | 52 |
| 5 | 5 I.U. chorionic gonadotrophin† | 73 | 42 | 42 | 97 | 51 | 48 |
| 8 | 5 R.U. pituitary gonadotrophin* + 5 I.U. chorionic gonadotrophin† | 59 | 48 | 19 | 107 | 55 | 49 |
| 7 | 5 R.U. pituitary gonadotrophin* + 50 γ estrone | 59 | 41 | 30 | 96 | 46 | 52 |
| 4 | 5 R.U. pituitary gonadotrophin* + 100 γ estrone | 54 | 41 | 24 | 81 | 32 | 60 |
| 7 | 5 R.U. pituitary gonadotrophin* + 500 γ testosterone propionate | 60 | 43 | 28 | 101 | 55 | 46 |
| 5 | 5 R.U. pituitary gonadotrophin* + 1 mg. testosterone propionate | 55 | 53 | 4 | 94 | 38 | 56 |
| 6 | 5 R.U. pituitary gonadotrophin* + 50 γ estrone + 500 γ testosterone propionate + 1 mg. progesterone | 67 | 56 | 16 | 97 | 51 | 48 |

* Ambinon 'Organon.'

† Pregnyl 'Organon.'

also has a favorable effect, 100 γ of estrone having more of an effect than 50 γ, but the effect of testosterone cannot be obtained. Careful examination discloses that the diminished weight loss is caused by the formation of new corpora lutea, since those corpora present originally are all decreased in size.

### TESTOSTERONE CAUSES FORMATION OF CAVITIES AND IS ANTAGONISTIC TO THE GRANULOSA

I mentioned a short while ago that the treatment of young rats with estrone only for 5 days increases the number of follicles, which are without cavities in the very young animals, and that the effect is similar in hypophysectomized animals. When chorionic gonadotrophin is administered simultaneously the follicles are not much larger but they contain cavities and these are very irregular, so-called "necrotic disintegration forms" (1). The cavities have a cystic character, the granulosa is loosened and partly destroyed and often forms only a thin layer instead of a many-layered cushion. We also noticed the atrophy of the theca which is otherwise absent.

Papanicolaou *et al.* (58), Selye and Collip (59), Greene, Burrill and Ivy (60), Bradbury *et al.* (61), Guyénot and Naville-Trolliet (62, 63) observed in guinea pigs that chorionic gonadotrophin makes the clitoris grow and that male substances must therefore have been produced. Paesi and Gaarenstroom (64) repeated the experiment in hypophysectomized rats with chorionic gonadotrophin and observed the same: the clitoris grew and its weight increased from 3–5 mg. to 9–10 mg. This is not a direct effect of the chorionic gonadotrophin upon the clitoris since this effect is absent in castrated rats.

De Jongh *et al.* make it very probable that abnormal formation of cavities and the disturbance in the granulosa are caused by male substances since they were able to bring about exactly the same condition by the combined administration of estrogens and testosterone. Changes both with chorionic gonadotrophin and with testosterone are still much clearer when the estrogens are first administered separately. Thus the cooperation of the two hormones, the female and the male, is of great formative importance for the structural development of the ovaries. They probably play a decisive part in the normal formation of the cavities of the graafian follicles and also in the development of a hypertrophy of the theca and this holds true especially for the androgens.

TABLE 9

*Production of Testosterone in the Ovaries of Hypophysectomized Young Rats*

| Number of rats | Status | Weight of animals | Treatment for 10 days | Clitoris weight |
|---|---|---|---|---|
| | | *gm.* | | *mg.* |
| 3 | Hypophysectomized | 52–66 | 10 I.U. chorionic gonadotrophin* | 9.5–10 |
| 2 | Hypophysectomized | 64–66 | NaCl 0.9% | 6.5– 7 |
| 4 | Hypophysectomized | 57–63 | 5 I.U. chorionic gonadotrophin* | 8.5–10.5 |
| 5 | Hypophysectomized | 57–65 | NaCl 0.9% | 4 – 4.5 |
| 2 | Hypophysectomized and castrated | 71–90 | 10 I.U. chorionic gonadotrophin* | 4 – 4.5 |
| 2 | Hypophysectomized and castrated | 69–77 | NaCl 0.9% | 4.5– 5 |
| 4 | Castrated | 45–51 | 5 I.U. chorionic gonadotrophin* | 3 – 5 |
| 4 | Castrated | 46–49 | NaCl 0.9% | 4.5– 4.5 |
| 2 | Normal | 52–58 | 1 mg. progesterone | 4 – 5 |
| 2 | Normal | 58–60 | Oil | 4 – 4.5 |

* Pregnyl ''Organon.''

The estrogens probably protect the ovum against the influence of the androgens and are of importance during the extrusion of the first polar body (Moricard (65)).

Does testosterone have an effect other than the formation of cavities?

Yes it does. The most interesting is the production of a complete ovulation. De Jongh *et al.*, on the basis of their concepts, administered 1 mg. of testosterone propionate daily for five consecutive days to 4 young mice weighing 6 grams. All animals exhibited enlargement of the ovaries caused by the presence of mature follicles with cavities. In one of the mice the egg could still be found in the neighborhood of the ruptured follicle. In three other mice treated in the same way only an enlargement of the ovary by ripening follicles was observed as was the case in this ovulating mouse.

### TESTOSTERONE AS INSTIGATOR OF OVULATION

Does ovulation actually have anything to do with testosterone? Certainly it does. In immature animals weighing 6 grams no ovulation ever occurs. Prior to this observation Shapiro and Zwarenstein (66) produced ovulation in toads by testosterone. This, however, did not prove a direct effect, and one could not speak of exclusive testosterone action since this occurred in the presence of the pituitary.

In order to understand this effect of testosterone let us consider an interesting observation (67). Testosterone causes single vascular buds of rat ovaries to grow into the follicles long before luteinization occurs, whereupon the bud widens and forms a dead end. The penetration occurs in a spot which is exactly opposite to that from which vessels grow into the tissue during luteinization; that is, the side opposite the egg, whereas in this case the vascular bud is located next to the egg. The vascular wall is very thin, and de Jongh *et al.* (67) believe that these effects are of decisive significance for normal ovulation. As long as 37 years ago, long before the age of hormones, von Winiwarter and Sainmont (68) observed a similar picture in a young 3-month-old cat. Twenty-eight years earlier, in 1881, Schulin (69) observed vascular buds penetrating the granulosa in the neighborhood of the egg cells in human ovaries. (Of course, it cannot be proven that the male substance formed in the ovaries causes this effect but the results described are indicative.)

The "hemorrhagic points" observed many thousands of times in the undamaged follicle in the Aschheim and Zondek reaction which remains unexplained so far becomes understandable. One also understands from where the blood comes during ovulation since a vascular thin follicular wall which ruptures did not offer any explanation. Furthermore one can understand the increase of the intrafollicular pressure which leads to rupture when terminal vessels grow into a closed cavity.

Let us briefly touch the question of the formation of the steroids in the ovary as well as at what site, at what time, and under what influences.

### SITE AND TIME OF PRODUCTION OF THE STEROIDS

Because of lack of time I have to restrict myself to a very sketchy summary of our, in part, still hypothetical concept, which was mainly developed by de Jongh *et al.* and also by Freud, concerning the interaction of the different sex hormones in the ovary. Regarding the rationale of this concept I shall have to refer you to the papers in the Nederlandsche Akademie van Wetenschappen and to the monograph appearing shortly in English (1). I shall therefore mention only very briefly when, where and under the influence of which gonadotrophins we believe the steroid hormones are produced and what their immediate effects are.

I must emphasize again that we do not know the chemical nature of the substances involved when we speak of "androgens," "estrogens" or "progestogens." This latter expression coined by Brent several years ago seems to me to be very suitable for general use.

During the first postnatal days, when the status of the rat can probably still be compared with the fetal condition of primates, hormones presumably play no role. The egg cells exert a directing influence on the ovariocytes, which are almost the only other cells present. Freud applies the term ovariocytes to all cells other than the egg cell and connective tissue cells, blood vessel cells, nerve-sheath cells and the external endothelial cells of the stratum germinativum. These ovariocytes can adopt during their lifetime very different functional stages, probably partly depending in which morphological context they occur.

TABLE 10

*Ovarian Morphology and Function (Rat)*

| Stimulus | Tissues acted upon | Effects | Growth effect | | | Progestational effect | |
|---|---|---|---|---|---|---|---|
| | | | Clit. | Ut. | Vag. | Ut. | Vag. |
| Egg | Ovariocytes | Avascular spherical layer<br>Solid sphere<br>Primary follicle | | | | | |
| GE (FSH?) | Egg | Activation<br>Protection against androgens | | | | | |
| GI (LH?) | Interstitial ovariocytes | Production of *androgens* | | | | | |
| GE + GI | Primary follicles | Secondary follicle (granulosa, theca interna)<br>Production of *estrogens* | | | | | |
| Androgens | Secondary follicles: granulosa | Cavity formation<br>Harmful to granulosa and egg | + | − | − | − | − |
| | Secondary follicles: theca | Vascular bud<br>Ovulation<br>Hemorrhagic points | | | | | |
| Estrogens | Granulosa | Maintenance and growth (mitoses) | − | + | + | − | − |
| GI + estrogens (+ androgens) | Theca + vessels | Luteinization<br>Corpus luteum (no living egg) | | | | | |
| Prolactin | Corpora lutea | Corpora lutea of pregnancy (hypertrophic cells)<br>Production of *progesterone* | | | | + | + |

They are influenced, other than by the egg cell, by the gonadotrophic hormones and also by the steroids produced locally under the stimulating effect of the gonadotrophins. One and the same ovariocyte can form part of the first single layer surrounding the egg, it can later be included in the granulosa, or also in the theca participating in its formation. It can furthermore take part in the formation of the corpus luteum, and later on atrophy and die. It can also revive and follow a course like that of any other ovariocyte derived from the so-called interstitium.

The cells pass thus through very different stages, described very appropriately in a monograph by Freud which I hope will soon appear in print as micro- (or normo-), macro- and hypertrophic. He calls the general tendency of trophic development eutrophy. Only the so-called eutrophic stages are normally capable of mitosis.

As mentioned before, the egg cells exert a directing influence and thus it is likely that the primordial egg organizes the monolayer membrane around itself. This mechanism is non-hormonal, as was demonstrated recently by Paesi of the laboratory at Leyden. Young rats show a decrease in number of all follicles 8 to 15 days after hypophysectomy with the exception of those of a diameter of about 23 to 33 μ, which are definitely increased. The membrane consists of a single layer of cuboid cells. These results have been obtained by counting and measuring 10 μ-thick slices of ovaries sectioned in series, counting either consecutive slices or slices at intervals, never more than 10 apart. Whether the egg cell, so to speak, stops short at giving the command: "Surround me!" or whether it also orders divisions, in other words, whether it influences mitoses in its environment, we do not know.

### PRODUCTION OF ANDROGENS

During the first five days of life of the rat, androgens are produced either spontaneously or under the stimulation of GI in the extremely numerous ovariocytes located outside the sphere of influence of the egg cell. Within the first 5 days by far the largest number of the approximately 20,000 egg cells present at birth have degenerated and only about 3,000 are still surrounded by their satellites. We deduce the production of androgens after the administration of chorionic gonadotrophin from the growth of the clitoris (58–64). Estrogens are not yet formed at this time of life, since the uterus and vagina remain entirely unchanged. This proves a certain insensibility of the ovariocytes of these very young animals.

### FORMATION OF ESTROGENS

However, estrogens are produced probably not much later and at least before the maturation of follicles has progressed very far. Before the production of estrogens and independent of hormonal influences, the egg cells continue to grow and produce an increase in thickness of the spherical layer. This occurs under the influence of GE, which promotes particularly the growth of the ovariocytes in epithelial arrangement. Thus the more syncytial structure of the granulosa is formed, probably preventing, together with the influence of the living egg, any deep penetration of blood vessels. Such a penetration into the theca, the formation of which has meanwhile started and which is more remote from the egg, is possible, because vessels have made their appearance in the meantime, penetrating the ovary from the hilus. Estrogens are probably formed before this in the "primordial form" in which granulosa and theca are still not separated; for 10-day-old animals, in which estrogens can be demonstrated show estrus effects after the administration of GI, which is impossible during the very first days, as mentioned before. These estrogens play a part in the process of maturation. It is only during the third week that a division of the "primordial form," just mentioned, takes place between an external layer containing oval nuclei and an internal layer having round nuclei. Thereupon the androgens penetrate the follicle, which grows under the influence of GE and of estrogens. The site of the continuous production of the androgens is unknown. They cause the formation of cavities in the follicles, which continue to be of regular shape. Sufficient GE maintains the growth of the granulosa, which, in the absence of GE, would perish together with the follicles forming so-called "necrotic distintegration forms." They accumulate probably in the cavity, flooding the egg or rather the corona radiata and are supposed to induce the first division of maturation according to Moricard, as mentioned before. The androgens enhance the development or the hypertrophy of the theca and furthermore the penetration of single vascular buds, which lead to ovulation and hemorrhage. After this has happened and when the influence of the egg has ceased because it has been expelled or died (through the influence of androgens which are produced too fast or in too large amounts under the stimulation of the artificially administered gonadotrophins) the syncytial structure of the granulosa and the border between it and the theca interna disappear. Vessels penetrate from the latter thereby converting the ovariocytes into lutein cells. Undoubtedly GI and estrogens cooperate in this process. The cooperation of androgens formed in increasing amounts under the influence of GI can, of course, not be excluded.

## PRODUCTION OF PROGESTERONE

Progesterone is produced in the luteinized cells after they have attained a certain size. In order to function it is most likely that prolactin is required—which represents, so to speak, a third group of gonadotrophins.

I need not explain to you at great length that these schematic concepts do not entirely describe what is actually happening. I hope that the new facts which I have presented have been of interest to you in connection with the facts with which you are already familiar.

I consider the following as relatively new and important: Certain steroids which, in their capacity as hormones, normally have an effect upon remote sites, undoubtedly have a direct and stimulating effect upon the gonads, in other words, those organs which mainly produce them. Up to now, very definite effects of testosterone upon the testes and of both this androgen and estrogens upon the ovary have been demonstrated. It is, therefore, extremely likely that these same steroids or related ones exert an influence during and after their production upon the reactive tissues. This action can be a particularly strong one since these substances are present in high concentration because of the proximity of the site of production and action.

I repeat that the facts known to date will continue to be of importance even if their theoretical interpretation should cease to be satisfactory. At this time it is advantageous to use this theory in order to recognize the entire context in the presence of so many details, and above all, to enable us to plan new experiments since, as I need hardly mention, so much is left unexplained.

## REFERENCES

1. Gaarenstroom, J. H., and de Jongh, S. E., Monograph on the effect of gonadotrophic and sex hormones on the gonads of rats, Amsterdam, Elsevier, 1946.
2. Smith, P. E., *Proc. Soc. Exper. Biol. & Med.*, 1926, 24, 131.
3. Aschheim, S., and Zondek, B., *Klin. Wchnschr.*, 1927, 6, 1322.
4. Fluhmann, C. F., *J.A.M.A.*, 1929, 93, 672.
5. Cole, H. H., and Hart, G. H., *Am. J. Physiol.*, 1930, 93, 57.
6. Guyénot, E., *Experimentia,* 1945, 1, 1.
7. Freud, J., and Dingemanse, E., *Acta brev. Neerland.*, 1941, 11, 37.
8. Laqueur, E., Hart, P. C., and de Jongh, S. E., *Proc. Nederland. Akad. Wetensch.*, 1926, 29, 591.

8a. Hamburger, C., Halvorsen, K., and Pedersen, J., *Acta Pharmacol.*, 1945, 1, 129.

9. Moore, C. R., *Proc. Second Intern. Cong. Sex Research* (1930), 1931, 293.
10. Lahr, J. H. F., *Acta brev. Neerland.*, 1940, 10, 59.
11. Chow, B. F., The chemistry and physiology of the hormones, 1944, 26.
12. Greep, R. O., van Dyke, H. B., and Chow, B. F., *J. Biol. Chem.*, 1940, 133, 289.
13. Greep, R. O., van Dyke, H. B., and Chow, B F., *Endocrinology,* 1942, 30, 635.
14. Chow, B. F., *Ann. N. Y. Acad. Sc.*, 1943, 43, 309.
15. Shedlovsky, T., Rothen, A., Greep, R. O., van Dyke, H. B., and Chow, B. F., *Science,* 1940, 92, 178.
16. Li, C. H., Simpson, M. E., and Evans, H. M., *J. Am. Chem. Soc.*, 1942, 64, 367.
17. Li, C. H., Evans, H. M., and Wonder, D. H., *J. Gen. Physiol.*, 1940, 23, 733.
18. Evans, H. M., Simpson, M. E., Tolksdorf, S., and Jensen, H., *Endocrinology,* 1939, 25, 529.
19. de Jongh, S. E., and Gaarenstroom, J. H., *Acta brev. Neerland.*, 1941, 11, 184.
20. Greep, R. O., *Endocrinology,* 1938, 23, 154.
21. Gaarenstroom, J. H., and de Jongh, S. E., *Proc. Nederland. Akad. Wetensch.*, 1943, 52, 446.

22. Laqueur, E., and Deelen, T., *Nederl. tijdschr. v. geneesk.*, 1936, 80, 743.
23. Albright, F., Parson, W., and Bloomberg, E., *J. Clin. Endocrinol.*, 1941, 1, 375.
24. Nietzsche, F. Also sprach Zarathustra, 1902, p. 47, Leipzig, C. S. Neumann.
24a. Gigon, A., and Buchs, S., *Bull. de l'Acad. Suisse des Sci. Méd.*, 1944, 1, 79.
25. Walsh, E. L., Cuyler, W. K., and McCullagh, D. R., *Am. J. Physiol.*, 1934, 107, 508.
26. Nelson, W. O., Symposium on hormones (Sigma Xi lecture), 1936–1937, pp. 378–393.
27. Cutuly, E., McCullagh, D. R., and Cutuly, E. C., *Am. J. Physiol.*, 1937, 119, 121.
28. Gaarenstroom, J. H., and Freud, J., *Acta brev. Neerland.*, 1938, 8, 178.
28a. Leathem, J. H., and Brent, B. J., *Proc. Soc. Exper. Biol. & Med.*, 1943, 52, 341.
29. Gaarenstroom, J. H., and de Jongh, S. E., *Arch. internat. de pharmacodyn. et de therap.*, 1941, 65, 206.
30. Westman, A., and Jacobsohn, D., *Acta path. et microbiol. Scandinav.*, 1938, 15, 445.
31. Hertz, R., and Meyer, R. K., *Endocrinology*, 1937, 21, 756.
32. Cutuly, E., McCullagh, D. R., and Cutuly, E., *Endocrinology*, 1937, 21, 241.
33. Moore, C. R., and Price, D., *Anat. Rec.*, 1938, 71, 59.
34. Nelson, W. O., *Am. J. Physiol.*, 1940, 129, p. 430.
35. Gaarenstroom, J. H., and de Jongh, S. E., *Acta brev. Neerland.*, 1940, 10, 77.
36. Evans, H. M., Pencharz, R. I., and Simpson, M. E., *Endocrinology*, 1934, 18, 601.
37. Smith, P. E., and Leonard, S. L., *Anat. Rec.*, 1934, 58, 145.
38. Bokslag, J. G. H., *Acta brev. Neerland.*, 1937, 7, 87.
39. Selye, H., *Proc. Soc. Exper. Biol. & Med.*, 1941, 46, 142.
39a. de Fremery, P., *Acta brev. Neerland.*, 1941, 11, 187.
40. Nelson, W. O., and Merckel, C. E., *Proc. Soc. Exper. Biol. & Med.*, 1938, 38, 737.
41. Scowen, E. F., *Anat. Rec.*, 1938, 70, Suppl. 3, 71.
42. Smith, P. E., and Brouha, L., Les hormones sexuelles, Paris, 1938, 201.
42a. Smith, P. E., *Yale J. Biol. & Med.*, 1944, 17, 281.
43. Dvoskin, S., *Proc. Soc. Exper. Biol. & Med.*, 1943, 54, 111.
43a. Masson, G., *Am. J. Med. Sc.*, 1945, 209, 324.
44. Laqueur, E., Dingemanse, E., Hart, P. C., and de Jongh, S. E., *Klin. Wchnschr.*, 1927, 6, 1859.
44a. Laqueur, E., *Verhdg. dtsch. Pharm. Ges. Wuerzburg*, 1927, 82.
44b. Freud, J., *Biochem. J.*, 1933, 27, 1438.

45. Selye, H., Collip, J. B., and Thomson, D. L., *Proc. Soc. Exper. Biol. & Med.*, 1935, **32**, 1377.
46. Hohlweg, W., *Klin. Wchnschr.*, 1934, **13**, 92.
47. Hohlweg, W., *Klin. Wchnschr.*, 1936, **15**, 1832.
48. David, K. G., Freud, J., and Uyldert, I. A., *Arch. internat. de pharmacodyn. et de therap.*, 1941, **65**, 259.
49. Hohlweg, W., *Klin. Wchschr.*, 1937, **16**, 586.
50. Gaarenstroom, J. H., *Proc. Nederland. Akad. Wetensch.*, 1942, **45**, 953.
51. David, K. G., Freud, J., and Uyldert, I. E., *Arch. internat. de pharmacodyn. et de therap.*, 1941, **65**, 312.
52. Selye, H., and Collip, J. B., *Endocrinology*, 1936, **20**, 667.
53. Pencharz, R. I., *Science*, 1940, **91**, 554.
53a. Simpson, M. E., Evans, H. M., Fraenkel-Conrat, H. L., and Li, C. H., *Endocrinology*, 1941, **28**, 37.
54. Williams, P. C., *Nature*, 1940, **145**, 388.
54a. Robson, J. M., *J. Physiol.*, 1937, **90**, 435.
Robson, J. M., *Quart. J. Exper. Physiol.*, 1938, **28**, 49.
55. Uyldert, I. E., David, K. G., and Freud, J., *Acta brev. Neerland.*, 1940, **10**, 105.
56. Uyldert, I. E., *Arch. internat. de pharmacodyn. et de therap.*, 1943, **69**, 114.
57. Clark, H. M., *Anat. Rec.*, 1935, **61**, 175.
58. Papanicolaou, G. N., and Falk, E. A., *Proc. Soc. Exper. Biol. & Med.*, 1934, **31**, 750.
59. Selye, H., and Collip, J. B., *Proc. Soc. Exper. Biol. & Med.*, 1933, **30**, 647.
60. Greene, R. R., Burrill, M. W., and Ivy, A. C., *Endocrinology*, 1939, **24**, 351.
61. Bradbury, J. T., and Gaensbauer, F., *Proc. Soc. Exper. Biol. & Med.*, 1939, **41**, 128.
62. Guyénot, E., Ponse, K., and Naville-Trolliet, I., *C. R. Acad. Sc.*, 1934, **198**, 1830.
63. Guyénot, E., and Naville-Trolliet, I., *Revue Suisse Zool.*, 1936, **43**, 415.
64. Paesi, F. J. A., and Gaarenstroom, J. H., *Nederland. Akad. Wetensch.*, 1943, **52**, 592.
65. Moricard, R., Facteurs hormonaux et cytoplasmiques de la division nucleaire Meiose et Gonadotrophines, Paris, 1940.
66. Shapiro, H. A., and Zwarenstein, H., *J. Physiol.*, 1937, **89**, 38P.
67. de Jongh, S. E., Gaarenstroom, J. H., and Paesi, F. J. A., *Nederland. Akad. Wetensch.*, 1944, **53**, 100.
68. von Winiwarter, H., and Sainmont, G., *Arch. de biol.*, 1909, **24**, 74.
69. Schulin, *Arch. f. mikrosk. Anat.*, 1881, **19**, 442.

**Tables 1 to 9 are essentially identical with those appearing in the monograph (1).**

# ENZYMATIC REACTIONS IN CARBOHYDRATE METABOLISM[1]

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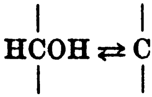
MORE than 18 years have elapsed since the writer had the privilege of presenting before the Harvey Society his analysis of the factors involved in carbohydrate metabolism. The large amount of new information that has come to light in the intervening years cannot be reviewed here. In general progress has been registered along two separate lines of investigation, those carried out on (more or less) intact animals and those carried out with isolated enzyme systems. In the former category, to mention only some of the more recent work, we find such important results as the recognition that the anterior pituitary (Houssay) and the adrenal cortex (Long) participate in the regulation of the blood sugar level, while work falling under the latter category has given us detailed knowledge of the intermediary reactions of carbohydrate metabolism. It will be the purpose of this presentation to examine how far these two lines of investigation can be integrated.

### EXPERIMENTS ON INTACT ANIMALS

Carbohydrate metabolism in the intact animal may be represented by a number of over-all reactions; the most important of these are:

(1) The reversible reaction, glycogen ⇄ lactic acid.
(2) The reversible reaction, glycogen ⇄ glucose.
(3) Oxidation of carbohydrate to $CO_2$ and $H_2O$.
(4) 
$\overset{|}{\underset{|}{\mathrm{HCOH}}} \rightleftarrows \overset{|}{\underset{|}{\mathrm{CH_2}}}$; interconversions, e.g., of carbohydrate carbon to fat carbon or of protein carbon to carbohydrate

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[1] Lecture delivered May 16, 1946.

carbon. These are explored most successfully by means of isotopic carbon.

Simultaneous determination of all these factors on the same animal has led to a type of experiment which is referred to as carbohydrate balance. It may be of interest to examine briefly what information concerning carbohydrate metabolism can be obtained from such balances.

Table 1 summarizes some of these experiments. The animals were first fasted for 24 hours, providing the basal values for groups A and B, while the basal values for group C were those obtained on group B after 4 hours of glucose absorption.

TABLE 1

*Changes in the Distribution of Glycogen in Rats*

Values are expressed in mg. per 100 gm. rat

| Experimental conditions | Glycogen in liver | Glycogen in rest of body | Carbohydrate oxidized | Blood sugar (per 100 cc.) | Number of animals |
|---|---|---|---|---|---|
| A. Fasted for 24 hours | | | | | |
| *Basal values* | *7* | *136* | | *92* | 16 |
| Fasted 48 hrs. | + 3 | − 25 | | − 8 | 21 |
| Insulin (3 hrs.) | − 2 | − 34 | 56 | − 12 | 4 |
| Epinephrine (3 hrs.) | + 36 | − 57 | | + 18 | 6 |
| Hypophysectomized (3 hrs.) | | − 36 | 60 | − 10 | 6 |
| B. After 4 hours of glucose absorption | | | | | |
| *Basal values* | *7* | *136* | | *92* | 16 |
| Controls | + 192 | + 263 | 465 | + 58 | 10 |
| Insulin | + 75 | + 393 | 550 | − 28 | 7 |
| Epinephrine | + 212 | + 136 | 357 | + 98 | 8 |
| C. 3-hour post-absorptive period | | | | | |
| *Basal values* | *220* | *432* | | *158* | 13 |
| Controls | − 49 | − 167 | 220 | − 45 | 4 |
| Insulin | − 141 | − 188 | 434 | − 89 | 4 |
| Epinephrine | + 26 | − 298 | 263 | + 16 | 5 |

From the changes in glycogen distribution observed after injection of epinephrine, in conjunction with other experimental evidence, it was deduced that muscle glycogen is converted to liver glycogen by way of blood lactic acid. This interpretation is supported by recent experiments of Stetten and Klein (1) on the uptake of deuterium into liver glycogen from deuterium oxide administered to the animals. They found that the newly formed liver glycogen after glucose feeding contained 38, after lactate feeding 57 and after epinephrine injection 56 per cent of the deuterium content of the body fluids, as compared to a theoretical maximum of 66 per cent. Glycogen synthesis from 3-carbon fragments would be expected to result in much more exchange of stably bound hydrogen than synthesis from glucose directly. The fact that the liver glycogen after epinephrine injection had the same isotope content as after lactate feeding supports the idea of the lactic acid cycle.

Other deductions which may be made from the experiments in table 1 are the following. Beginning with group A, it may be seen that fasting is characterized by a rigid economy of the carbohydrate reserves; there is hardly any change in the glycogen content of rats between 24 and 48 hours of fasting. After hypophysectomy the animals are losing their carbohydrate reserves much more rapidly than the normal animals; in fact, they lose more carbohydrate during 3 hours of fasting than do normal animals in 24 hours of fasting. The hypophysectomized animals resemble in this respect normal fasting animals injected with insulin. The indications are that the anterior pituitary exerts a restraining influence on carbohydrate metabolism and that this as well as a diminished secretion of insulin, represent regulatory mechanisms which come into play during fasting.

Indications of such a regulatory mechanism can be seen when one compares the amount of carbohydrate oxidized during glucose absorption in group B and in the post-absorptive period in group C. As the fast continues, carbohydrate oxidation is restricted more and more until it reaches a minimum which does not exceed appreciably the new formation of carbohydrate from other sources and this allows the glycogen content of the tissues to remain constant.

Injection of insulin during glucose absorption causes an increased utilization of sugar in the peripheral tissues. Here insulin is merely superimposed upon the animals' own, presumably optimal insulin secretion. During the post-absorptive period, injection of insulin causes a marked disturbance of the normal regulatory mechanism. Liver glycogen disappears 3 times as rapidly as in the control animals, while muscle glycogen is not much affected. At the same time the restrictive influences on carbohydrate oxidation which are characteristic for the post-absorptive period are removed and the animals are now oxidizing sugar at the same rate as during glucose absorption. The interpretation given to these experiments is that insulin is favoring a reaction concerned with the utilization of blood sugar in the tissues and that the liver is breaking down glycogen more rapidly in order to meet the increased demand for blood sugar.

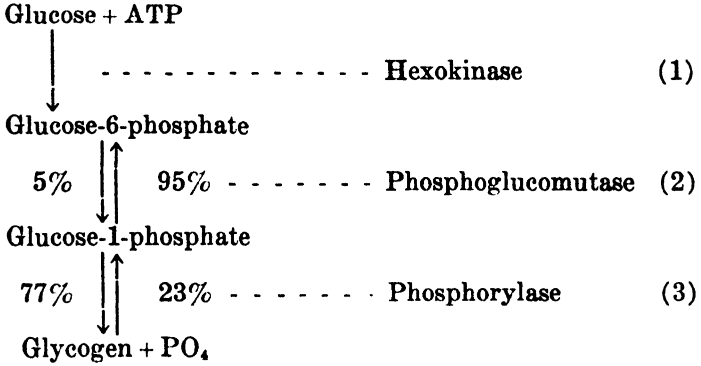
What reaction might be favored by insulin was not revealed by these experiments and after some consideration it was concluded that another approach was necessary in order to shed some light on this problem. Work on the whole animal was given up in favor of a study of individual enzymatic reactions. This involved the extraction of enzymes from the tissues and their separation from each other. In the course of this work several enzymes were crystallized, in particular phosphorylase (2), the enzyme which catalyzes the breakdown and synthesis of glycogen in the tissues.

## EXPERIMENTS WITH ISOLATED ENZYME SYSTEMS

It was shown that the over-all reaction, glucose ⟶ glycogen, which is characteristic for the whole animal may be resolved into the following enzymatic steps (3).

The first reaction, catalyzed by hexokinase, an enzyme originally discovered by Meyerhof in yeast, and now known to occur in all animal tissues, is largely irreversible. In this reaction ATP (adenosinetriphosphate) is expended and this raises the problem of the regeneration of ATP.

The equilibria for the second and third reaction at pH 7, as determined for each enzyme acting separately, are shown above. Although the position of the equilibrium of the second reaction appears to be unfavorable, the overall reaction will nevertheless proceed in the direction of glycogen synthesis as long as inorganic phosphate is being removed as fast as it is formed in the phosphorylase reaction. In the test tube this can be accomplished by the addition of barium ions which decrease the solubility of phosphate; in the intact animal the removal of inorganic phosphate is accomplished by oxidation of carbohydrate which is



coupled with the regeneration of ATP and thus permits ATP to be used again in the hexokinase reaction.

The over-all reaction, glycogen ⇄ lactic acid, has also been resolved into individual enzymatic steps, and these are embodied in the well-known Embden-Meyerhof scheme. Wood and collaborators (4) have found that glucose isolated from liver glycogen was labelled with heavy carbon in positions 3 and 4, when they administered glucose and $NaHC^{13}O_3$ to fasting rats; labelling of these positions would be predicted from a reversal of the reactions of the Embden-Meyerhof scheme, following the incorporation of labelled carbon in the reaction, pyruvate + $CO_2$ ⇄ oxaloacetate.

One of the important results emerging from this work is the realization that carbohydrate oxidation is an offspring of the chain of reactions which lead from glycogen or from glucose to lactic acid. It is now generally agreed that carbohydrate oxidation at the pyruvate level is initiated by a condensation reaction between a $C_2$ and $C_4$ carbon compound; one oxidative cycle, $(C_2 + C_4) \longrightarrow 2CO_2 + 2H_2O + C_4$, regenerates the $C_4$ compound (oxaloacetate) which can then again react with the $C_2$ fragment derived from pyruvic acid. Here again experiments with carbon isotopes have given strong support to the general validity of a scheme of carbohydrate oxidation proposed by Krebs, although minor modifications of the original scheme appear necessary. The primary condensation product between $C_2$ and $C_4$ has not been identified, but assuming it to be a member of the tricarboxylic acid group, all further oxidative steps are known.

A greatly simplified scheme of carbohydrate metabolism, leaving out many of the intermediate steps, may now be presented (figure 1). The main feature of this scheme, as far as this discussion is concerned, is that glucose in order to be metabolized must first react with ATP. Once glucose is converted to glucose-6-phosphate by the hexokinase reaction, several metabolic pathways are open. Glucose may become glycogen, it may be converted to fat, or it may be oxidized, and the magnitude of each transformation will depend on enzymatic equilibria determined by the metabolic state of the animal. In a previously fasted rat about 50 per cent of the absorbed glucose is deposited as glycogen in liver and muscle, while the rest is oxidized (table 1), but once the

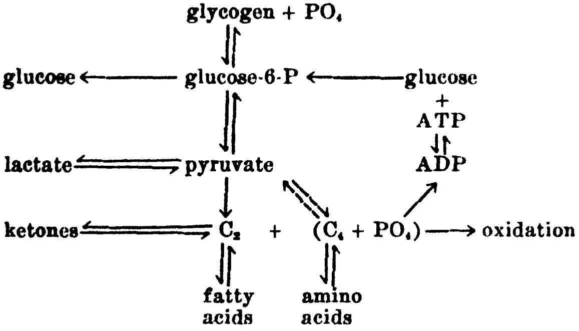


FIG. 1. Simplified scheme of carbohydrate metabolism.

glycogen stores are replenished, a large part of the absorbed glucose may be deposited as fat.

Blood sugar formation in the liver is due to the action of a specific phosphatase which converts glucose-6-phosphate to glucose and inorganic phosphate. In muscle such a phosphatase is absent and owing to this fact muscle contributes lactate instead of glucose to the blood stream. In both liver and muscle the concentration of inorganic phosphate within the cells should have a decisive influence on whether the reactions will proceed in the direction of glycogen synthesis or glycogen breakdown.

Similar effects of the concentration of other reacting substances exist. For example, the fate of the reactive 2-carbon fragments formed from fatty acids in the liver depends on the availability of $C_4$ dicarboxylic acid (Lehninger (5)); in their absence the fragments condense with each other to form acetoacetate, in their presence the $C_2$ intermediate is oxidized.[2] This oxidation, as shown in the scheme, is coupled with the uptake of inorganic phosphate and the regeneration of ATP (6).

It seemed likely that the regulation of the blood sugar level depends on special tissue structures sensitive to changes in the blood sugar concentration and on the release of hormones which would act on one or more of the enzymatic reactions discussed above. One example may be cited here, namely, the secretion of insulin by the islet tissue of the pancreas which is known to respond to the blood sugar level. The action of insulin in the intact animal has been investigated extensively, and since most of the intermediary reactions of carbohydrate metabolism are known, one would think that the mechanism of action of insulin would be amenable to investigation.

Krebs and Eggleston (7) have shown that the addition of insulin to minced pigeon breast muscle could under certain conditions prolong the oxygen consumption of these preparations.

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[2] Since the liver is the main site of formation of ketone bodies, it should be pointed out that the antiketogenic mechanism in the liver depends upon three reactions which lead to the formation of dicarboxylic acid, namely, pyruvate + $CO_2 \rightleftarrows$ oxaloacetate, transamination between pyruvate and either glutamate or aspartate, and direct deamination of these two amino acids.

Their results have been confirmed by several investigators. In an over-all reaction of this type, composed of a large number of enzymatic steps which cannot be separated from each other, there would be considerable difficulty in finding out at what point in the chain of reactions insulin was exerting its effect. At least, this was the conclusion reached with respect to some observations made by Colowick and Sutherland in our laboratory in 1942 on cat liver dispersions. In contrast to the system investigated by Krebs, the rate of oxygen consumption was increased from the start when insulin was added. One of several rather striking experiments is shown in figure 2. The dialyzed homogenates were deficient with respect to adenine nucleotides, as shown by the effect of addition of adenylic acid. Without and with added adenylic acid insulin produced a very marked effect on the $O_2$ consumption. The experiments have not been published so far,

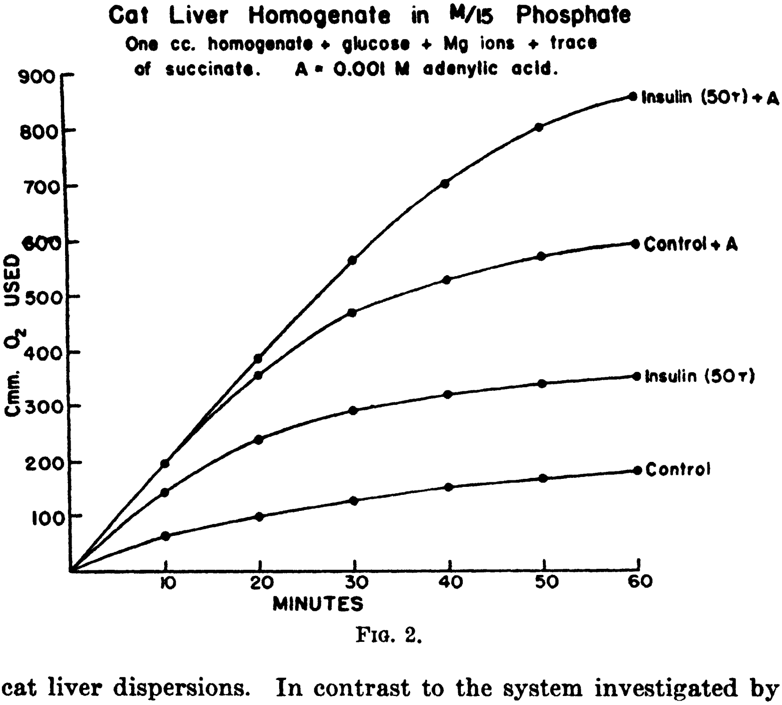

FIG. 2.

because in spite of much effort it has not been possible to control the system sufficiently to make the insulin effect regularly reproducible on different liver preparations, in itself an indication that the mechanism of action of insulin in this system is not understood.

From time to time, when individual enzymatic reactions were being investigated, the effect of addition of insulin was tried, but a clear-cut effect on an isolated enzyme system in vitro could not be demonstrated. Although this led to the suggestion that the action of insulin and other hormones may depend on a more or less intact cell structure, the search for an in vitro effect of insulin was continued.

### YEAST AND ANIMAL HEXOKINASE

A number of circumstances permitted us to investigate the hexokinase reaction. Colowick and Kalckar (8) had shown with hexokinase prepared from baker's yeast that this enzyme catalyzed a one-step reaction; only one of the two labile phosphate groups of ATP (adenosinetriphosphate) was transferred to glucose. ADP (adenosinediphosphate) could not act as phosphate donor. They also showed that glucose was phosphorylated on carbon atom 6, giving rise to the formation of glucose-6-phosphate. Both points have recently been shown to apply also to brain hexokinase. The hexokinase reaction may therefore be written:

$$\text{glucose} + \text{ATP} \rightarrow \text{glucose-6-phosphate} + \text{ADP}.$$

Colowick and Kalckar also developed a manometric method for the determination of hexokinase activity which is based on the fact that one acid equivalent is formed for each phosphate group transferred from ATP to glucose. Enzyme activity can also be followed chemically by determining the disappearance of glucose or of ATP. In most experiments to be discussed later the reaction was followed manometrically and a terminal glucose analysis was made on the contents of the Warburg vessels.

Yeast hexokinase has been prepared in crystalline form. This enzyme needs $Mg^{++}$ for its activity as does the corresponding animal enzyme, but no other co-factor is needed. The animal hexokinase has so far been purified only partially. Hexokinase activity has been demonstrated in our laboratory in extracts of liver, kidney, brain, skin, intestine, anterior pituitary, heart and skeletal muscle. In the case of muscle and other tissues the hexokinase activity found was of sufficient magnitude to account for the rate of metabolism of glucose observed in these tissues in the intact animal.

Colowick and Price (9) have carried out a detailed study of rat muscle hexokinase. They found that previous difficulties in getting an active hexokinase preparation from muscle was due to the acid reaction of water extracts, pH about 6.2. At this pH rapid inactivation of hexokinase occurred, but in investigating this phenomenon they found that enzyme activity could be restored by addition of dihydrocozymase. When the extracts were aged at pH 6 and dialyzed, the necessity for yet another co-factor was discovered, namely, guanine. In such extracts either component alone was without effect, but when both guanine and dihydrocozymase were added hexokinase activity was restored.

### EFFECT OF PITUITARY AND ADRENAL CORTEX EXTRACTS AND INSULIN OF THE HEXOKINASE REACTION[3]

Referring again to the simplified scheme of carbohydrate metabolism in figure 1, it may be seen that hexokinase initiates the utilization of glucose in the tissues and that if the activity of this enzyme were enhanced by insulin, most of the known effects of insulin in the intact animal could be explained. In spite of many trials an accelerating effect of insulin on the hexokinase reaction could not be demonstrated.

It then seemed possible that the clue to the situation might be in the reverse argument, namely, that if hexokinase were inhibited in the intact animal, hyperglycemia and glycosuria would result. With this in mind, experiments were carried out with muscle hexokinase preparations from rats made diabetic with alloxan.

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[3] The experimental work reported in this section was carried out by S. P. Colowick, G. T. Cori, W. H. Price and M. W. Slein to whom should go the credit for these observations (10, 11).

It was observed that in some cases the manometric measurements indicated a lag period of about 10 minutes duration before hexokinase activity set in and that addition of insulin abolished the lag period. Such an experiment is reproduced in figure 3.

Remembering the experiments of Houssay which demonstrated an amelioration of diabetes following the removal of the pituitary, we made the guess that the lag period may be due to an inhibitory pituitary factor present in the tissue extracts of alloxan diabetic rats. If this were so, the effect of insulin would consist in the removal of this inhibition. That a pituitary factor could cause the inhibition was suggested by the observation that curves for hexokinase activity similar to those shown in figure 3 were obtained in muscle extracts of normal rats previously injected with pituitary or in muscle extracts to which an isoelectric protein fraction of the pituitary was added in vitro (figure 4). Here again insulin counteracted the pituitary inhibition.

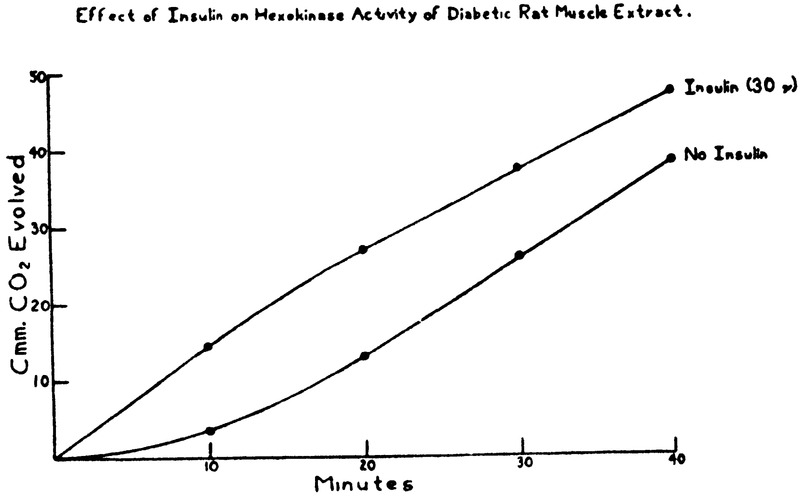
Effect of Insulin on Hexokinase Activity of Diabetic Rat Muscle Extract.

FIG. 3.

The short duration of the inhibition in figures 3 and 4 was attributed to the rapid destruction or inactivation of the pituitary factor in crude tissue extracts. In order to eliminate some of the destructive factors, the hexokinase in muscle extract was purified by fractionation with acetone. A much more prolonged inhibition could be produced by addition of pituitary to such purified extracts (figure 5). Similar results were obtained when hexokinase prepared from an acetone powder of calf brain was used as the test system.

In view of the experiments reported by Long on the amelioration of diabetes after removal of the adrenals, it was of interest to study the effect of adrenal cortex extract. Figure 5 shows that a commercial extract (Upjohn), by itself without effect, increased the inhibitory action of anterior pituitary on a purified hexokinase preparation. This effect of adrenal cortex extract is also seen in crude muscle extracts in which the action of pituitary alone is of short duration (figure 6).

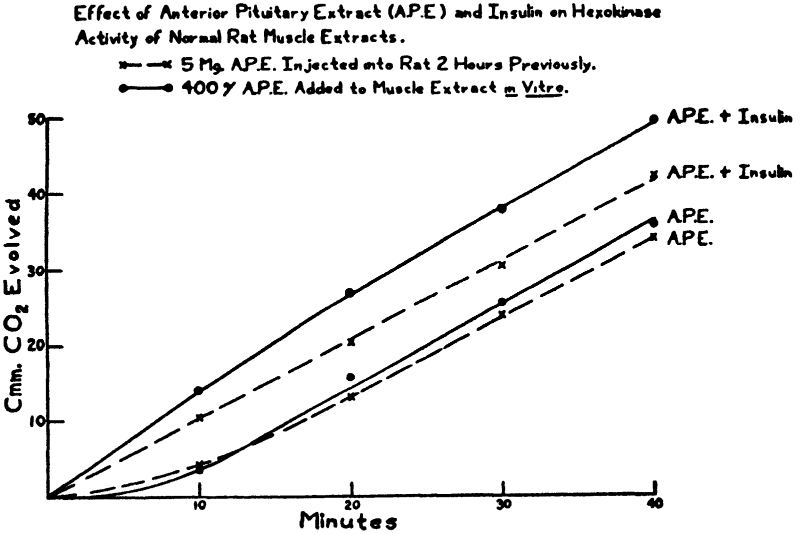

FIG. 4.

The effect of adrenal cortex preparations has been made use of in an analysis of the hexokinase activity in crude muscle extracts of alloxan diabetic rats. It may be assumed that tissue extracts of normal animals contain insulin, and hence the hexokinase is free to act even if some pituitary factor is present; only if an excess of pituitary is added can an inhibition be demonstrated. In extracts of diabetic rats there should be a preponderance of the pituitary factor over insulin, because of the destruction of the islet tissue by alloxan. It would follow that if the extracts of diabetic rats contain the pituitary factor and no insulin, addition of adrenal cortex extract alone should produce inhibition. This is shown in figure 7. Without addition of adrenal cortex extract there is a short lag period, with cortex extract added a marked inhibition occurs which in this case amounts to about 70 per cent for the 30-minute period, when compared with the sample to which insulin was added.

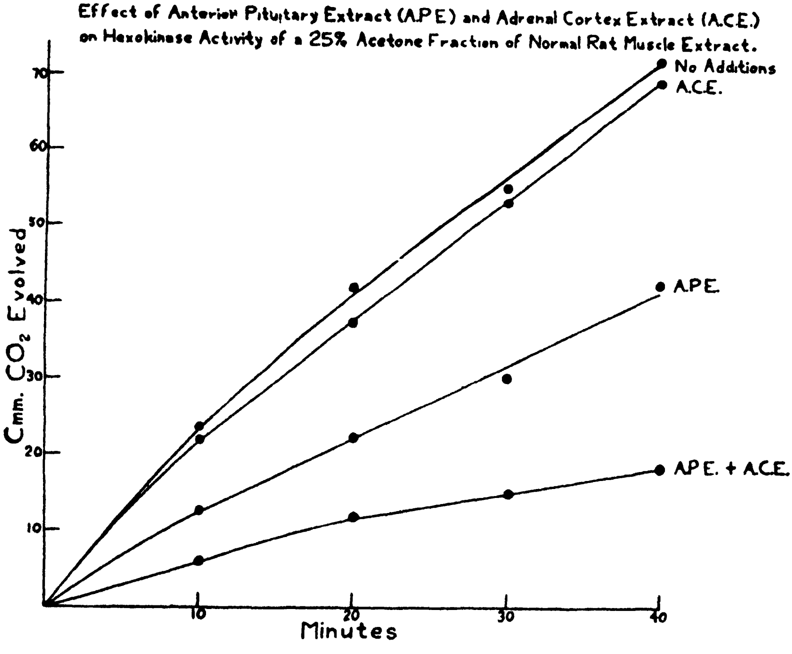

FIG. 5.

It should be emphasized that among 34 diabetic extracts investigated only 50 per cent showed a strong inhibition on addition of destructive factors, the hexokinase in muscle extract was purified by fractionation with acetone. A much more prolonged inhibition could be produced by addition of pituitary to such purified extracts (figure 5). Similar results were obtained when hexokinase prepared from an acetone powder of calf brain was used as the test system.

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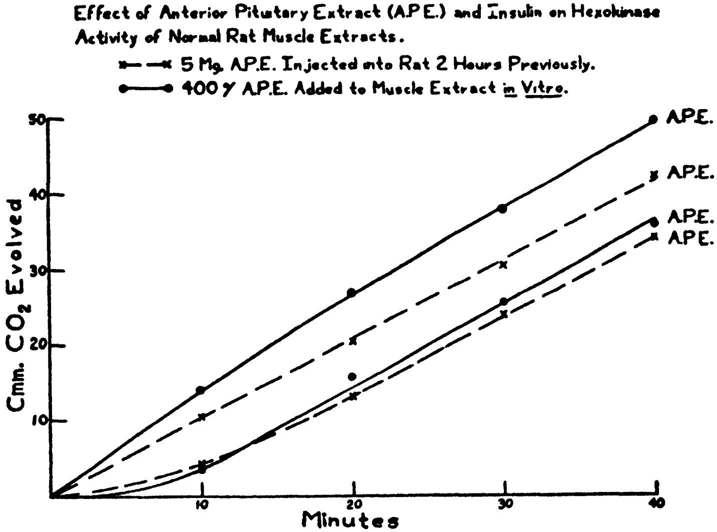

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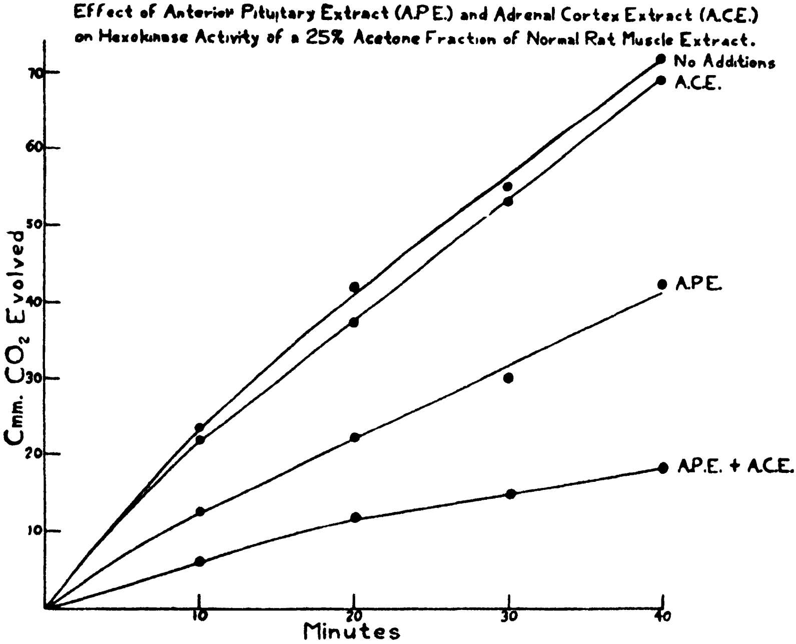

FIG. 5.

It should be emphasized that among 34 diabetic extracts investigated only 50 per cent showed a strong inhibition on addition of adrenal cortex extract alone and some gave completely negative results. This is probably due to several factors. Not all rats injected with alloxan get equally diabetic. If some islet tissue remained intact, the tissue extracts would contain insulin. Similarly, the lack of effect of adrenal cortex preparations alone on extracts from normal rats may be due either to less pituitary factor being present, or what seems more likely, to the presence of enough insulin to overcome the inhibition. Another factor is the instability of the inhibitory substance. When extracts from diabetic rats were kept for 1 to 2 hours at 0°, addition of cortex extract no longer produced inhibition, although the same extracts had given a positive result when tested immediately after preparation.[4] Finally, the optimal conditions for the extraction of the inhibitor from the tissues have not been worked out. The difficulty here is that both the inhibitor and the hexokinase must be extracted at the same time.

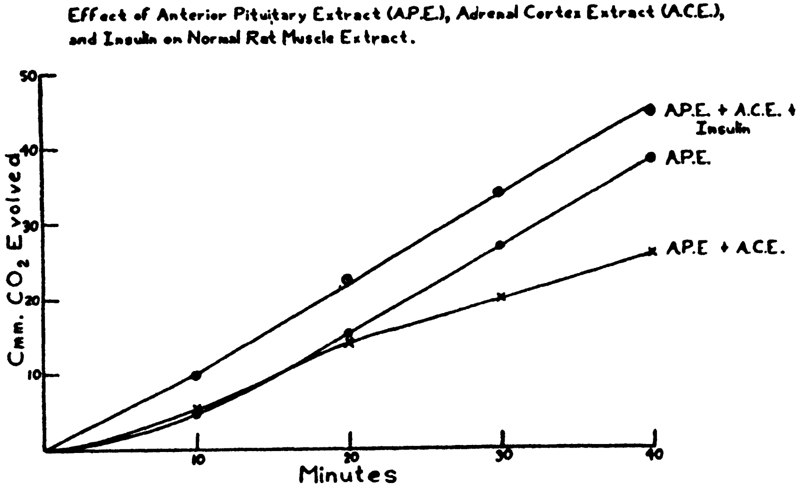

FIG. 6.

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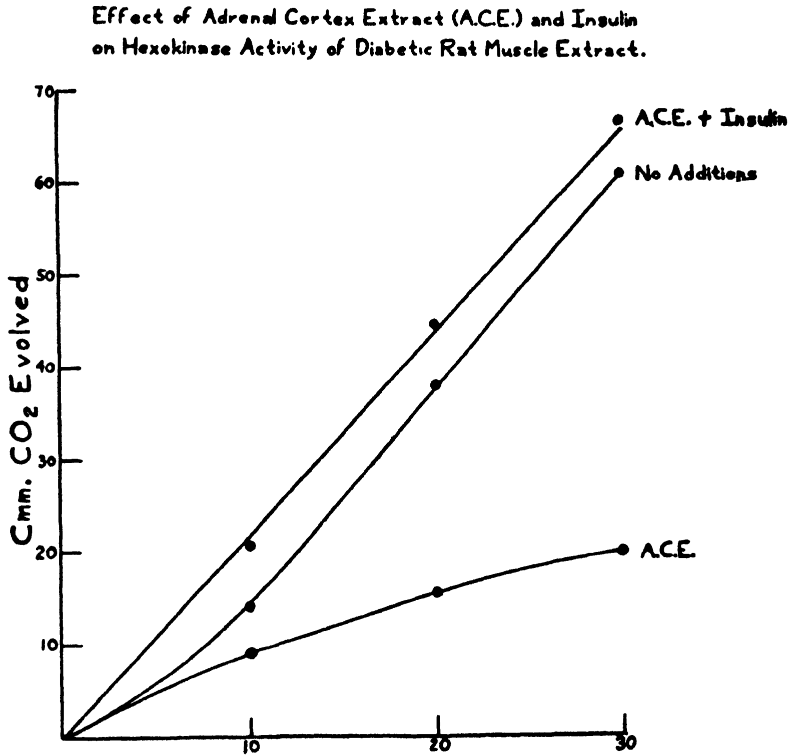
[4] This recalls the interesting observations of Shorr (12), who found that muscle strips prepared from depancreatized animals showed initially an inhibition of glucose oxidation but that this inhibition disappeared when the tissues were kept for some hours in vitro under aerobic conditions.

Further experiments with muscle extracts from diabetic rats are shown in figure 8. Addition of 10 micrograms of insulin had a suboptimal effect, while 50 micrograms completely released the inhibition caused by 0.1 cc. of adrenal cortex extract added to 2.5 cc. of reaction mixture. Next it was determined that addition of 0.05 cc. of cortex extract had as much inhibitory effect as 0.1 cc., while 0.01 cc. was suboptimal. The inhibitory action of the Upjohn extract could be reproduced with an amorphous fraction of the adrenal cortex, kindly supplied to us by Dr. Kendall and Dr. Kuizenga, but not with the crystalline compounds A, B and



Effect of Adrenal Cortex Extract (A.C.E.) and Insulin on Hexokinase Activity of Diabetic Rat Muscle Extract.

FIG. 7.

E of Kendall. The nature of the adrenal substance which causes this inhibition has not been determined.

The instability of the pituitary factor has so far prevented progress in its purification. Pituitaries from various species (beef, sheep, pig, horse) have yielded active extracts. It is essential that the glands be obtained as soon as possible after the death of the animals and that they be frozen immediately. The glands

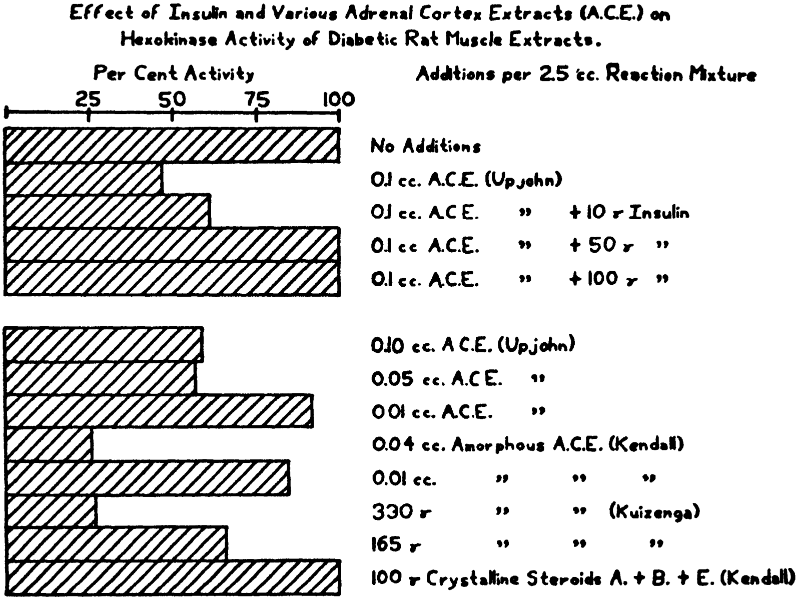


FIG. 8.

may be extracted for 30 minutes with dilute barium hydroxide, pH 10, followed by removal of barium with dilute sulfuric acid and separation of the isoelectric precipitate formed between pH 6.3 and 5.8. This precipitate, containing about 10 to 15 per cent of the extracted proteins, does not show any hexokinase activity of its own and inhibits brain hexokinase in the presence of adrenal cortex extract. The glands may also be extracted with dilute sulfuric acid at pH 5.7, followed by adjustment to pH

5.3 and dilution. The precipitate formed on dilution contains most of the activity. Adsorption on aluminum hydroxide has effected further purification. Lyophilized glands have occasionally yielded active material.

All the preparations so far obtained are unstable in solution and generally lose their activity when kept for 1 to 2 hours in an ice-bath. They could not be lyophilized or frozen in solution without loss of activity. It has not been possible so far to determine the nature of the destructive factors. Acetone fractionation could not be used because it caused inactivation of the pituitary factor.

A method of preparation which is successful in the majority of cases will be reported in detail. At the present stage of purification it did not seem profitable to try to identify the pituitary inhibitor of hexokinase with other factors known to be present in anterior pituitary extracts. A number of these factors have been tested on the hexokinase system with negative results. This would rule out unspecific inhibitory effects, as would the effect of insulin in counteracting an existing inhibition. It may be mentioned here that this insulin effect is perfectly reproducible and that when insulin is reduced by cysteine or inactivated by treatment with alkali, it is no longer capable of counteracting the pituitary inhibition. Assuming that one is dealing with an interaction of proteins, a final analysis of the system would require that all three proteins, the enzyme, the inhibitor and the releasing agent, be available in relatively pure form.

## DISCUSSION

The over-all reaction, glycogen $\rightleftarrows$ lactic acid, is not inhibited by the anterior pituitary fractions which inhibit the hexokinase reaction. There is also no inhibition of the reaction, glucose-6-phosphate $\rightarrow$ glucose + phosphate, which occurs in the liver. Reference to figure 1 will show that the hexokinase reaction is not involved in either case. This would explain why lactate, pyruvate, alanine and other amino acids are convertible to glucose in the liver of depancreatized dogs and why injection of epinephrine in such dogs causes an excretion of extra sugar in the urine. In the latter case blood lactic acid derived from muscle glycogen would be converted to glucose in the liver.

Lukens (13) investigated the resynthesis of glycogen in previously stimulated muscles of normal and depancreatized cats. The amount of glycogen lost on stimulation was essentially the same in both types of animals and the initial resynthesis of glycogen, presumably from hexosemonophosphate and lactate (which accumulate in muscle during stimulation) occurred at the same rate. However, the final restitution of the glycogen level which depends on the uptake of blood sugar by the muscles was delayed considerably in the depancreatized animals. The results of Lukens could therefore be explained by an inhibition of the hexokinase reaction in the diabetic animals.

Another observation which fits into this scheme is that of Bueding and coworkers (14). They showed that the rise in blood pyruvate which is seen in normal animals following the injection of glucose is absent in depancreatized dogs and that injection of insulin is necessary to bring back the increase in blood pyruvate in diabetic dogs. The normal animal responds with an increase in blood pyruvate, because the reactions leading to its formation occur somewhat faster than the reactions causing a removal of pyruvate. The removal of injected pyruvate from the blood occurs at the same rate in normal and diabetic dogs. It is also known that the muscles of diabetic dogs can convert their glycogen to pyruvate and lactate as rapidly as those of normal animals. As shown in figure 1, the lack of response of blood pyruvate to glucose injection could therefore be due to an inhibition of the hexokinase reaction and the effect of insulin to a release of this inhibition.

The idea that removal or destruction of the islet tissue of the pancreas results in under-utilization of blood sugar has recently received strong support in the experiments of Stetten and Klein (1) with deuterium. They found that in alloxan diabetic rats about 75 per cent of the urinary glucose was of dietary origin and that only 25 per cent represented gluconeogenesis. That certain amino acids, because they are convertible to glucose, are the source of gluconeogenesis, has long been accepted, and since there is an increased nitrogen excretion in the urine of depancreatized dogs, such animals convert more protein to sugar than do normal animals. In this sense there exists an overproduction of sugar in the diabetic animal, but this is of minor importance in comparison to the primary disturbance in carbohydrate metabolism which is in the utilization of blood sugar. Houssay's and Long's experiments have shown that this impairment of blood sugar utilization is due to a preponderance of anterior pituitary and adrenal cortex hormones over insulin. The present experiments would explain these phenomena by the action of anterior pituitary, adrenal cortex and insulin on hexokinase, an enzyme directly concerned with blood sugar utilization.

One more point deserves discussion here. The scheme in figure 1 indicates that a $C_2$ intermediate is formed from both carbohydrate and fat which is then oxidized over a common pathway, a fact which is in itself of great importance. Such oxidation cannot result in a gain of carbohydrate. A net gain would result, however, if a $C_2$ fragment derived from fatty acids could be converted to pyruvate (by reductive carboxylation) or if two such fragments could condense to form a dicarboxylic acid, since the latter, as shown in the scheme, is convertible to carbohydrate. So far these reactions have not been observed in the animal body. The isotope studies with tracer carbon show interconversions between all three foodstuffs, carbohydrate, fat and protein, because the carbon fragments pass through a common metabolic pool, but they have given no indication that fatty acids are a source of gluconeogenesis (15).

A good deal of additional work will be required before a complete integration between experiments on the whole animal and on isolated enzyme systems can be achieved. It is possible that other points of action of anterior pituitary and insulin exist. The oxidation of glucose can be inhibited in the intact animal by the injection of a pituitary factor prepared by Greaves (16), and the oxidation can be accelerated by insulin in vitro, as shown in the experiment in figure 2. Finally, insulin may also have a direct stimulatory effect on some enzymatic reaction, as indicated by the fact that it produces hypoglycemia in hypophysectomized, adrenalectomized animals.

These possibilities are mentioned here in order to indicate the point of view that the writer has adopted, namely, that so far only a beginning has been made in the understanding of the mechanism of action by which hormones regulate the rate of enzymatic reactions in the body.

## REFERENCES

1. Stetten, D., Jr., and Klein, B. V., *J. Biol Chem.*, 1945, 159, 593.
2. Green, A. A., and Cori, G. T., *J. Biol. Chem.*, 1943, 151, 21.
3. Colowick, S. P., and Sutherland, E. W., *J. Biol. Chem.*, 1942, 144, 423.
4. Wood, H. G., Lifson, N., and Lorber, V., *J. Biol. Chem.*, 1945, 159, 475.
5. Lehninger, A. L., *J. Biol. Chem.*, 1945, 161, 413.
6. Colowick, S. P., Kalckar, H. M., and Cori, C. F., *J. Biol. Chem.*, 1941, 137, 343.
7. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, 1938, 32, 913.
8. Colowick, S. P., and Kalckar, H. M., *J. Biol. Chem.*, 1943, 148, 117.
9. Colowick, S. P., and Price, W. H., *J. Biol. Chem.*, 1945, 157, 415; 159, 563.
10. Price, W. H., Cori, C. F., and Colowick, S. P., *J. Biol. Chem.*, 1945, 160, 633.
11. Price, W. H., Slein, M. W., Colowick, S. P., and Cori, G. T., *Federation Proc.*, 1946, 5, 150.
12. Shorr, E., *Cold Spring Harbor Symposia*, 1939, 7, 323.
13. Lukens, F. D. W., *Ann. Int. Med.*, 1934, 8, 727.
14. Bueding, E., Fazekas, J. F., Herrlich, H., and Himwich, H. E., *J. Biol. Chem.*, 1943, 148, 97.
15. Buchanan, J. M., Sakami, W., Gurin, S., and Wilson, D. W., *J. Biol. Chem.*, 1945, 159, 695.
16. Greaves, J. D., Freiberg, I. K., and Johns, H. E., *J. Biol. Chem.*, 1940, 133, 243.

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